MODULATION OF DIACYLGLYCEROL ACYLTRANSFERASE 2 EXPRESSION

FIELD OF THE INVENTION

The present invention provides compositions and methods for modulating the expression of diacylglycerol acyltransferase 2. In particular, this invention relates to compounds, particularly oligonucleotide compounds, which, in preferred embodiments, hybridize with nucleic acid molecules encoding diacylglycerol acyltransferase 2. Such compounds are shown herein to modulate the expression of diacylglycerol acyltransferase 2.

BACKGROUND OF THE INVENTION

Triglycerides are one of the major energy storage molecules in eukaryotes. The absorption of triglycerides (also called triacylglycerols) from food is a very efficient process which occurs by a series of steps wherein the dietary triacylglycerols are hydrolyzed in the intestinal lumen and then resynthesized within enterocytes. The resynthesis of triacylglycerols can occur via the monoacylglycerol pathway which commences with monoacylglycerol acyltransferase (MGAT) catalyzing the synthesis of diacylglycerol from monoacylglycerol and fatty actyl-CoA. An alternative synthesis of diacylglycerols is provided by the glycerol-phosphate pathway which describes the coupling of two

molecules of fatty acyl-CoA to glycerol-3-phosphate. In either case, diacylglycerol is then acylated with another molecule of fatty acyl-CoA in a reaction catalyzed by one of two diacylglycerol acyltransferase enzymes to form the triglyceride (Farese et al., *Curr. Opin. Lipidol.*, **2000**, *11*, 229-234).

The reaction catalyzed by diacylglycerol acyltransferase is the final and only committed step in triglyceride synthesis. As such, diacylglycerol acyltransferase is involved in intestinal fat absorption, lipoprotein assembly, regulating plasma triglyceride concentrations, and fat storage in adipocytes. The first diacylglycerol acyltransferase, diacylglycerol transferase 1, was identified in 1960 and the human and mouse genes encoding this protein were isolated in 1998 (Cases et al., *Proc. Natl. Acad. Sci. U. S. A.*, **1998**, *95*, 13018-13023; Oelkers et al., *J. Biol. Chem.*, **1998**, *273*, 26765-26771). Mice lacking diacylglycerol acyltransferase 1 are viable and can still synthesize triglycerides through other biological routes, suggesting the existence of multiple mechanisms for triglyceride synthesis (Smith et al., *Nat. Genet.*, **2000**, *25*, 87-90).

A second diacylglycerol transferase, diacylglycerol transferase 2 (also known as DGAT2, diacylglycerol O-transferase 2, acyl-CoA:diacylglycerol acyltransferase 2), was subsequently identified in the fungus Mortierella, humans and mice (Cases et al., *J. Biol. Chem.*, **2001**, *276*, 38870-38876; Lardizabal et al., *J. Biol. Chem.*, **2001**, *276*, 38862-38869). Enzymatic assays indicate that this recently identified protein does possess diacylglycerol transferase activity that utilizes a broad range of long chain fatty acyl-CoA substrates (Cases et al., *J. Biol. Chem.*, **2001**, *276*, 38870-38876).

Diacylglycerol transferase 2 is a member of a family of genes whose sequences are unrelated to diacylglycerol transferase 1. In addition to differing in sequence compared to diacylglycerol transferase 1, *in vitro* assays illustrate that diacylglycerol transferase 2 has higher activity at lower concentrations of magnesium chloride and oleoyl-CoA (Cases et al., *J. Biol. Chem.*, **2001**, *276*, 38870-38876). The predicted protein sequence of diacylglycerol transferase 2 contains at least one putative transmembrane domain, three potential N-linked glycosylation sites, six potential protein kinase C phosphorylation consensus sites, as well as sequences in common with a putative glycerol phosphorylation site found in acyltransferase enzymes (Cases et al., *J. Biol. Chem.*, **2001**, *276*, 38870-38876). The International Radiation Hybrid Mapping Consortium has mapped human diacylglycerol transferase 2 to chromosome 11q13.3.

In human tissues, the highest levels of diacylglycerol transferase 2 are detected in liver and white adipose tissues, with lower levels found in mammary gland, testis and peripheral blood leukocytes (Cases et al., *J. Biol. Chem.*, **2001**, *276*, 38870-38876). Two mRNA species of 2.4 and 1.8 kilobases are detected in human tissues, whereas the major diacylglycerol transferase 2 mRNA species in mouse tissues is 2.4 kilobases. In addition to liver and white adipose tissues, diacylglycerol transferase 2 is expressed in all segments of the small intestine in mice, with higher expression in the proximal intestine and lower expression in the distal intestine (Cases et al., *J. Biol. Chem.*, **2001**, *276*, 38870-38876).

Diacylglycerol transferase activity exhibits distinct patterns during postnatal development of the rat liver. As there is no correlation between the mRNA expression and activity patterns, post-translational modifications may

participate in the regulation of diacylglycerol transferase 2 activity during rat development (Waterman et al., *J. Lipid. Res.*, **2002**, *43*, 1555-1562).

Diacylglycerol transferase 2 mRNA is preferentially upregulated by insulin treatment, as shown by in vitro assays measuring the diacylglycerol activity from the membrane fraction of cultured mouse adipocytes (Meegalla et al., *Biochem. Biophys. Res. Commun.*, **2002**, *298*, 317-323). In fasting mice, diacylglycerol transferase 2 expression is greatly reduced, and dramatically increases upon refeeding. The expression patterns of two enzymes that participate in fatty acid synthesis, acetyl-CoA carboxylase and fatty acid synthase, respond to fasting and refeeding in a similar fashion. These results, combined with the observation that diacylglycerol transferase 2 is abundantly expressed in liver, suggest that diacylglycerol transferase 2 is tightly linked to the endogenous fatty acid synthesis pathway (Meegalla et al., *Biochem. Biophys. Res. Commun.*, **2002**, *298*, 317-323).

Studies of mice harboring a disruption in the diacylglycerol acyltransferase 1 gene provide evidence that diacylglycerol acyltransferase 2 contributes to triglyceride synthesis. Levels of diacylglycerol transferase 2 mRNA expression are similar in intestinal segments from both wild type and diacylglycerol transferase 1-deficient mice (Buhman et al., *J. Biol. Chem.*, **2002**, *277*, 25474-25479). Using magnesium chloride to distinguish between diacylglycerol transferase 1 and 2 activity, Buhman, et al. observed that, in diacylglycerol transferase 1-deficient mice, diacylglycerol transferase activity is reduced to 50% in the proximal intestine and to 10-15% in the distal intestine (Buhman et al., *J. Biol. Chem.*, **2002**, *277*, 25474-25479).

Additionally, diacylglycerol transferase 2 mRNA levels are not up-regulated the liver or adipose tissues of diacylglycerol transferase 1-deficient mice, even after weeks of high-fat diet (Cases et al., *J. Biol. Chem.*, **2001**, *276*, 38870-38876; Chen et al., *J. Clin. Invest.*, **2002**, *109*, 1049-1055). However, in ob/ob mice, which have a mutation in the leptin gene that results in obesity, diacylglycerol transferase 2 is more highly expressed than in wild type mice, suggesting that diacylglycerol transferase 2 may be partly responsible for the highly accumulated fat mass seen in these mice. Furthermore, the combined mutations of leptin and diacylglycerol transferase 1 leads to a three-fold elevation in diacylglycerol transferase 2 expression in white adipose tissue, compared to the levels in the same tissue from diacylglycerol transferase 1-deficient mice (Chen et al., *J. Clin. Invest.*, **2002**, *109*, 1049-1055). Diacylglycerol transferase 2 mRNA is also upregulated in the skin of these mice (Chen et al., *J. Clin. Invest.*, **2002**, *109*, 175-181). These data suggest leptin normally downregulates diacylglycerol transferase 2 expression, and that the upregulation of diacylglycerol transferase 2 in white adipose tissue in these mice may provide an alternate pathway for the triglyceride synthesis that still occurs in leptin deficient/ diacylglycerol transferase 1-deficient mice (Chen et al., *J. Clin. Invest.*, **2002**, *109*, 1049-1055).

Diacylglycerol acyltransferase 1 knockout mice exhibit interesting phenotypes in that they are lean, resistant to diet-induce obesity, have decreased levels of tissue triglycerides and increased sensitivity to insulin and leptin (Chen et al., *J. Clin. Invest.*, **2002**, *109*, 1049-1055; Smith et al., *Nat. Genet.*, **2000**, *25*, 87-90). As diacylglycerol transferase 2 also participates in triglyceride synthesis,

interfering with diacylglycerol transferase 2 may similarly lead to reduced body fat content.

The US pre-grant publications 20030124126 and 20020119138 claim and disclose a nucleic acid molecule encoding human diacylglycerol transferase 2 alpha, as well as compositions, including antisense oligonucleotides, for modulating the activity of said diacylglycerol transferase 2 alpha (Cases et al., **2003**).

The US pre-grant publication 20030104414 discloses and claims nucleic acid sequences which are members of a group of genes referred to as "protein cluster V" as well as the method for identification of an agent capable of modulating nucleic acid molecules in the protein cluster V group. This application also discloses the use of RNA interference or double-stranded RNA to disrupt the function of protein cluster V gene family members (Attersand, **2003**).

The US pre-grant publication 20030100480 discloses that diacylcglycerol transferase activity, including that of diacylglycerol transferase 2, may be modified by a variety of methods, including antisense, RNA interference and diacylglycerol transferase 2 antisense plasmid constructs (Smith et al., **2003**).

The US pre-grant publication 20030028923 claims and discloses a method for modifying the triacylglyerol composition in a plant cell, comprising transforming a plant cell with a nucleic acid construct encoding an enzyme active in the formation of triacylglycerol from diacylglycerol and fatty acyl substrates, including nucleic acid constructs in the antisense orientation. Also disclosed and claimed is a method for ameliorating a disease or condition associated with altered diacylglycerol acyltransferase activity by administering to a subject a therapeutically effective amount of a diacylglycerol acyltransferase agonist. This

application discloses that such antagonists can include antisense molecules (Lardizabal et al., **2003**).

The PCT publication WO 00/78961 claims and discloses isolated nucleic acid molecules selected from a group including a nucleic acid sequence encoding diacylglycerol acyltransferase 2. This publication also discloses that sense or antisense oligonucleotides binding to target nucleic acid sequences can interfere with transcription or translation of the disclosed and claimed nucleic acid molecules (Baker et al., **2000**).

Disclosed and claimed in the PCT publication WO 01/77389 are polynucleotides selected from a group of sequences including a nucleotide sequence encoding a human diacylglycerol acyltransferase. Also claimed and disclosed are a method for screening for the altered expression of said polynucleotides and a method for screening a library of molecules that specifically bind to said polynucleotide sequences (Shiffman et al., **2001**).

The PCT publication WO 01/68848 discloses and claims a nucleic acid molecules encoding secreted and transmembrane polypeptides, including a human diacylglycerol acyltransferase 2 nucleic acid molecule, and oligonucleotide probes derived from any of these sequences (Baker et al., **2001**).

Disclosed and claimed in the European patent application EP 1 308 459 are a group of polynucleotide sequences, including a nucleic acid molecule encoding human diacylglycerol acyltransferase 2, and antisense polynucleotides against this group of polynucleotide sequences (Isogai et al., **2003**).

The PCT publication WO 02/08260 discloses and claims an isolated, purified polynucleotide sequence with identity to a human diacylglycerol transferase 2 nucleotide sequence. This

application also discloses a substantially purified oligonucleotide that includes a region of nucleotide sequence that hybridizes to at least 8 consecutive nucleotides of sense or antisense sequence of a nucleotide sequence selected from a group consisting of sequences with identity to human diacylglycerol acyltransferase 2 (Botstein et al., **2002**).

Currently, there are no known therapeutic agents which effectively inhibit the synthesis of diacylglycerol acyltransferase 2. Consequently, there remains a long felt need for additional agents capable of effectively inhibiting diacylglycerol acyltransferase 1 function.

Antisense technology is an effective means for reducing the expression of specific gene products and may therefore prove to be uniquely useful in a number of therapeutic, diagnostic, and research applications for the modulation of diacylglycerol acyltransferase 2 expression.

The present invention provides compositions and methods for modulating diacylglycerol acyltransferase 2 expression.

SUMMARY OF THE INVENTION

The present invention is directed to compounds, especially nucleic acid and nucleic acid-like oligomers, which are targeted to a nucleic acid encoding diacylglycerol acyltransferase 2, and which modulate the expression of diacylglycerol acyltransferase 2. Pharmaceutical and other compositions comprising the compounds of the invention are also provided. Further provided are methods of screening for modulators of diacylglycerol acyltransferase 2 and methods of modulating the expression of diacylglycerol acyltransferase 2 in cells, tissues or animals comprising contacting said cells, tissues or animals with one or more of the compounds

or compositions of the invention. Methods of treating an animal, particularly a human, suspected of having or being prone to a disease or condition associated with expression of diacylglycerol acyltransferase 2 are also set forth herein. Such methods comprise administering a therapeutically or prophylactically effective amount of one or more of the compounds or compositions of the invention to the person in need of treatment.

DETAILED DESCRIPTION OF THE INVENTION

A. Overview of the Invention

The present invention employs compounds, preferably oligonucleotides and similar species for use in modulating the function or effect of nucleic acid molecules encoding diacylglycerol acyltransferase 2. This is accomplished by providing oligonucleotides which specifically hybridize with one or more nucleic acid molecules encoding diacylglycerol acyltransferase 2. As used herein, the terms "target nucleic acid" and "nucleic acid molecule encoding diacylglycerol acyltransferase 2" have been used for convenience to encompass DNA encoding diacylglycerol acyltransferase 2, RNA (including pre-mRNA and mRNA or portions thereof) transcribed from such DNA, and also cDNA derived from such RNA. The hybridization of a compound of this invention with its target nucleic acid is generally referred to as "antisense". Consequently, the preferred mechanism believed to be included in the practice of some preferred embodiments of the invention is referred to herein as "antisense inhibition." Such antisense inhibition is typically based upon hydrogen bonding-based hybridization of oligonucleotide strands or segments such that at least one strand or segment is cleaved, degraded, or otherwise rendered inoperable. In this regard,

it is presently preferred to target specific nucleic acid molecules and their functions for such antisense inhibition.

The functions of DNA to be interfered with can include replication and transcription. Replication and transcription, for example, can be from an endogenous cellular template, a vector, a plasmid construct or otherwise. The functions of RNA to be interfered with can include functions such as translocation of the RNA to a site of protein translation, translocation of the RNA to sites within the cell which are distant from the site of RNA synthesis, translation of protein from the RNA, splicing of the RNA to yield one or more RNA species, and catalytic activity or complex formation involving the RNA which may be engaged in or facilitated by the RNA. One preferred result of such interference with target nucleic acid function is modulation of the expression of diacylglycerol acyltransferase 2. In the context of the present invention, "modulation" and "modulation of expression" mean either an increase (stimulation) or a decrease (inhibition) in the amount or levels of a nucleic acid molecule encoding the gene, e.g., DNA or RNA. Inhibition is often the preferred form of modulation of expression and mRNA is often a preferred target nucleic acid.

In the context of this invention, "hybridization" means the pairing of complementary strands of oligomeric compounds. In the present invention, the preferred mechanism of pairing involves hydrogen bonding, which may be Watson-Crick, Hoogsteen or reversed Hoogsteen hydrogen bonding, between complementary nucleoside or nucleotide bases (nucleobases) of the strands of oligomeric compounds. For example, adenine and thymine are complementary nucleobases which pair through the formation of hydrogen bonds. Hybridization can occur under varying circumstances.

An antisense compound is specifically hybridizable when binding of the compound to the target nucleic acid interferes with the normal function of the target nucleic acid to cause a loss of activity, and there is a sufficient degree of complementarity to avoid non-specific binding of the antisense compound to non-target nucleic acid sequences under conditions in which specific binding is desired, i.e., under physiological conditions in the case of *in vivo* assays or therapeutic treatment, and under conditions in which assays are performed in the case of *in vitro* assays.

In the present invention the phrase "stringent hybridization conditions" or "stringent conditions" refers to conditions under which a compound of the invention will hybridize to its target sequence, but to a minimal number of other sequences. Stringent conditions are sequence-dependent and will be different in different circumstances and in the context of this invention, "stringent conditions" under which oligomeric compounds hybridize to a target sequence are determined by the nature and composition of the oligomeric compounds and the assays in which they are being investigated.

"Complementary," as used herein, refers to the capacity for precise pairing between two nucleobases of an oligomeric compound. For example, if a nucleobase at a certain position of an oligonucleotide (an oligomeric compound), is capable of hydrogen bonding with a nucleobase at a certain position of a target nucleic acid, said target nucleic acid being a DNA, RNA, or oligonucleotide molecule, then the position of hydrogen bonding between the oligonucleotide and the target nucleic acid is considered to be a complementary position. The oligonucleotide and the further DNA, RNA, or oligonucleotide molecule are complementary to each other when a sufficient number of complementary positions in each

molecule are occupied by nucleobases which can hydrogen bond with each other. Thus, "specifically hybridizable" and "complementary" are terms which are used to indicate a sufficient degree of precise pairing or complementarity over a sufficient number of nucleobases such that stable and specific binding occurs between the oligonucleotide and a target nucleic acid.

It is understood in the art that the sequence of an antisense compound need not be 100% complementary to that of its target nucleic acid to be specifically hybridizable. Moreover, an oligonucleotide may hybridize over one or more segments such that intervening or adjacent segments are not involved in the hybridization event (e.g., a loop structure or hairpin structure). It is preferred that the antisense compounds of the present invention comprise at least 70%, or at least 75%, or at least 80%, or at least 85% sequence complementarity to a target region within the target nucleic acid, more preferably that they comprise at least 90% sequence complementarity and even more preferably comprise at least 95% or at least 99% sequence complementarity to the target region within the target nucleic acid sequence to which they are targeted. For example, an antisense compound in which 18 of 20 nucleobases of the antisense compound are complementary to a target region, and would therefore specifically hybridize, would represent 90 percent complementarity. In this example, the remaining noncomplementary nucleobases may be clustered or interspersed with complementary nucleobases and need not be contiguous to each other or to complementary nucleobases. As such, an antisense compound which is 18 nucleobases in length having 4 (four) noncomplementary nucleobases which are flanked by two regions of complete complementarity with the target nucleic acid would have 77.8% overall complementarity with the target

nucleic acid and would thus fall within the scope of the present invention. Percent complementarity of an antisense compound with a region of a target nucleic acid can be determined routinely using BLAST programs (basic local alignment search tools) and PowerBLAST programs known in the art (Altschul et al., *J. Mol. Biol.*, **1990**, 215, 403-410; Zhang and Madden, *Genome Res.*, **1997**, 7, 649-656).

Percent homology, sequence identity or complementarity, can be determined by, for example, the Gap program (Wisconsin Sequence Analysis Package, Version 8 for Unix, Genetics Computer Group, University Research Park, Madison WI), using default settings, which uses the algorithm of Smith and Waterman (Adv. Appl. Math., 1981, 2, 482-489). In some preferred embodiments, homology, sequence identity or complementarity, between the oligomeric and target is between about 50% to about 60%. In some embodiments, homology, sequence identity or complementarity, is between about 60% to about 70%. In preferred embodiments, homology, sequence identity or complementarity, is between about 70% and about 80%. In more preferred embodiments, homology, sequence identity or complementarity, is between about 80% and about 90%. In some preferred embodiments, homology, sequence identity or complementarity, is about 90%, about 92%, about 94%, about 95%, about 96%, about 97%, about 98%, or about 99%.

B. Compounds of the Invention

According to the present invention, compounds include antisense oligomeric compounds, antisense oligonucleotides, ribozymes, external guide sequence (EGS) oligonucleotides, alternate splicers, primers, probes, and other oligomeric compounds which hybridize to at least a portion of the target nucleic acid. As such, these compounds may be introduced in

the form of single-stranded, double-stranded, circular or hairpin oligomeric compounds and may contain structural elements such as internal or terminal bulges or loops. Once introduced to a system, the compounds of the invention may elicit the action of one or more enzymes or structural proteins to effect modification of the target nucleic acid. One non-limiting example of such an enzyme is RNAse H, a cellular endonuclease which cleaves the RNA strand of an RNA:DNA duplex. It is known in the art that single-stranded antisense compounds which are "DNA-like" elicit RNAse H. Activation of RNase H, therefore, results in cleavage of the RNA target, thereby greatly enhancing the efficiency of oligonucleotide-mediated inhibition of gene expression. Similar roles have been postulated for other ribonucleases such as those in the RNase III and ribonuclease L family of enzymes.

While the preferred form of antisense compound is a single-stranded antisense oligonucleotide, in many species the introduction of double-stranded structures, such as double-stranded RNA (dsRNA) molecules, has been shown to induce potent and specific antisense-mediated reduction of the function of a gene or its associated gene products. This phenomenon occurs in both plants and animals and is believed to have an evolutionary connection to viral defense and transposon silencing.

The first evidence that dsRNA could lead to gene silencing in animals came in 1995 from work in the nematode, *Caenorhabditis elegans* (Guo and Kempheus, *Cell*, **1995**, 81, 611-620). Montgomery et al. have shown that the primary interference effects of dsRNA are posttranscriptional (Montgomery et al., *Proc. Natl. Acad. Sci. USA*, **1998**, *95*, 15502-15507). The posttranscriptional antisense mechanism defined in *Caenorhabditis elegans* resulting from exposure to

double-stranded RNA (dsRNA) has since been designated RNA interference (RNAi). This term has been generalized to mean antisense-mediated gene silencing involving the introduction of dsRNA leading to the sequence-specific reduction of endogenous targeted mRNA levels (Fire et al., *Nature*, **1998**, 391, 806-811). Recently, it has been shown that it is, in fact, the single-stranded RNA oligomers of antisense polarity of the dsRNAs which are the potent inducers of RNAi (Tijsterman et al., *Science*, **2002**, *295*, 694-697).

The oligonucleotides of the present invention also include variants in which a different base is present at one or more of the nucleotide positions in the oligonucleotide. For example, if the first nucleotide is an adenosine, variants may be produced which contain thymidine, guanosine or cytidine at this position. This may be done at any of the positions of the oligonucleotide. These oligonucleotides are then tested using the methods described herein to determine their ability to inhibit expression of diacylglycerol acyltransferase 2 mRNA.

In the context of this invention, the term "oligomeric compound" refers to a polymer or oligomer comprising a plurality of monomeric units. In the context of this invention, the term "oligonucleotide" refers to an oligomer or polymer of ribonucleic acid (RNA) or deoxyribonucleic acid (DNA) or mimetics, chimeras, analogs and homologs thereof. This term includes oligonucleotides composed of naturally occurring nucleobases, sugars and covalent internucleoside (backbone) linkages as well as oligonucleotides having non-naturally occurring portions which function similarly. Such modified or substituted oligonucleotides are often preferred over native forms because of desirable properties such as, for example, enhanced cellular uptake, enhanced affinity for a target nucleic acid and increased stability in the presence

of nucleases.

While oligonucleotides are a preferred form of the compounds of this invention, the present invention comprehends other families of compounds as well, including but not limited to oligonucleotide analogs and mimetics such as those described herein.

The compounds in accordance with this invention preferably comprise from about 8 to about 80 nucleobases (i.e. from about 8 to about 80 linked nucleosides). One of ordinary skill in the art will appreciate that the invention embodies compounds of 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, or 80 nucleobases in length.

In one preferred embodiment, the compounds of the invention are 12 to 50 nucleobases in length. One having ordinary skill in the art will appreciate that this embodies compounds of 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 nucleobases in length.

In another preferred embodiment, the compounds of the invention are 15 to 30 nucleobases in length. One having ordinary skill in the art will appreciate that this embodies compounds of 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 nucleobases in length.

Particularly preferred compounds are oligonucleotides from about 12 to about 50 nucleobases, even more preferably those comprising from about 15 to about 30 nucleobases.

Antisense compounds 8-80 nucleobases in length comprising a stretch of at least eight (8) consecutive

nucleobases selected from within the illustrative antisense compounds are considered to be suitable antisense compounds as well.

Exemplary preferred antisense compounds include oligonucleotide sequences that comprise at least the 8 consecutive nucleobases from the 5'-terminus of one of the illustrative preferred antisense compounds (the remaining nucleobases being a consecutive stretch of the same oligonucleotide beginning immediately upstream of the 5'-terminus of the antisense compound which is specifically hybridizable to the target nucleic acid and continuing until the oligonucleotide contains about 8 to about 80 nucleobases). Similarly preferred antisense compounds are represented by oligonucleotide sequences that comprise at least the 8 consecutive nucleobases from the 3'-terminus of one of the illustrative preferred antisense compounds (the remaining nucleobases being a consecutive stretch of the same oligonucleotide beginning immediately downstream of the 3'-terminus of the antisense compound which is specifically hybridizable to the target nucleic acid and continuing until the oligonucleotide contains about 8 to about 80 nucleobases). One having skill in the art armed with the preferred antisense compounds illustrated herein will be able, without undue experimentation, to identify further preferred antisense compounds.

C. Targets of the Invention

"Targeting" an antisense compound to a particular nucleic acid molecule, in the context of this invention, can be a multistep process. The process usually begins with the identification of a target nucleic acid whose function is to be modulated. This target nucleic acid may be, for example, a cellular gene (or mRNA transcribed from the gene) whose

expression is associated with a particular disorder or disease state, or a nucleic acid molecule from an infectious agent. In the present invention, the target nucleic acid encodes diacylglycerol acyltransferase 2.

The targeting process usually also includes determination of at least one target region, segment, or site within the target nucleic acid for the antisense interaction to occur such that the desired effect, e.g., modulation of expression, will result. Within the context of the present invention, the term "region" is defined as a portion of the target nucleic acid having at least one identifiable structure, function, or characteristic. Within regions of target nucleic acids are segments. "Segments" are defined as smaller or sub-portions of regions within a target nucleic acid. "Sites," as used in the present invention, are defined as positions within a target nucleic acid.

Since, as is known in the art, the translation initiation codon is typically 5'-AUG (in transcribed mRNA molecules; 5'-ATG in the corresponding DNA molecule), the translation initiation codon is also referred to as the "AUG codon," the "start codon" or the "AUG start codon". A minority of genes have a translation initiation codon having the RNA sequence 5'-GUG, 5'-UUG or 5'-CUG, and 5'-AUA, 5'-ACG and 5'-CUG have been shown to function *in vivo*. Thus, the terms "translation initiation codon" and "start codon" can encompass many codon sequences, even though the initiator amino acid in each instance is typically methionine (in eukaryotes) or formylmethionine (in prokaryotes). It is also known in the art that eukaryotic and prokaryotic genes may have two or more alternative start codons, any one of which may be preferentially utilized for translation initiation in a particular cell type or tissue, or under a particular set of conditions. In the context of the invention, "start

codon" and "translation initiation codon" refer to the codon or codons that are used *in vivo* to initiate translation of an mRNA transcribed from a gene encoding diacylglycerol acyltransferase 2, regardless of the sequence(s) of such codons. It is also known in the art that a translation termination codon (or "stop codon") of a gene may have one of three sequences, i.e., 5'-UAA, 5'-UAG and 5'-UGA (the corresponding DNA sequences are 5'-TAA, 5'-TAG and 5'-TGA, respectively).

The terms "start codon region" and "translation initiation codon region" refer to a portion of such an mRNA or gene that encompasses from about 25 to about 50 contiguous nucleotides in either direction (i.e., 5' or 3') from a translation initiation codon. Similarly, the terms "stop codon region" and "translation termination codon region" refer to a portion of such an mRNA or gene that encompasses from about 25 to about 50 contiguous nucleotides in either direction (i.e., 5' or 3') from a translation termination codon. Consequently, the "start codon region" (or "translation initiation codon region") and the "stop codon region" (or "translation termination codon region") are all regions which may be targeted effectively with the antisense compounds of the present invention.

The open reading frame (ORF) or "coding region," which is known in the art to refer to the region between the translation initiation codon and the translation termination codon, is also a region which may be targeted effectively. Within the context of the present invention, a preferred region is the intragenic region encompassing the translation initiation or termination codon of the open reading frame (ORF) of a gene.

Other target regions include the 5' untranslated region (5'UTR), known in the art to refer to the portion of an mRNA

in the 5' direction from the translation initiation codon, and thus including nucleotides between the 5' cap site and the translation initiation codon of an mRNA (or corresponding nucleotides on the gene), and the 3' untranslated region (3'UTR), known in the art to refer to the portion of an mRNA in the 3' direction from the translation termination codon, and thus including nucleotides between the translation termination codon and 3' end of an mRNA (or corresponding nucleotides on the gene). The 5' cap site of an mRNA comprises an N7-methylated guanosine residue joined to the 5'-most residue of the mRNA via a 5'-5' triphosphate linkage. The 5' cap region of an mRNA is considered to include the 5' cap structure itself as well as the first 50 nucleotides adjacent to the cap site. It is also preferred to target the 5' cap region.

Although some eukaryotic mRNA transcripts are directly translated, many contain one or more regions, known as "introns," which are excised from a transcript before it is translated. The remaining (and therefore translated) regions are known as "exons" and are spliced together to form a continuous mRNA sequence. Targeting splice sites, i.e., intron-exon junctions or exon-intron junctions, may also be particularly useful in situations where aberrant splicing is implicated in disease, or where an overproduction of a particular splice product is implicated in disease. Aberrant fusion junctions due to rearrangements or deletions are also preferred target sites. mRNA transcripts produced via the process of splicing of two (or more) mRNAs from different gene sources are known as "fusion transcripts". It is also known that introns can be effectively targeted using antisense compounds targeted to, for example, DNA or pre-mRNA.

It is also known in the art that alternative RNA transcripts can be produced from the same genomic region of DNA. These alternative transcripts are generally known as "variants". More specifically, "pre-mRNA variants" are transcripts produced from the same genomic DNA that differ from other transcripts produced from the same genomic DNA in either their start or stop position and contain both intronic and exonic sequence.

Upon excision of one or more exon or intron regions, or portions thereof during splicing, pre-mRNA variants produce smaller "mRNA variants". Consequently, mRNA variants are processed pre-mRNA variants and each unique pre-mRNA variant must always produce a unique mRNA variant as a result of splicing. These mRNA variants are also known as "alternative splice variants". If no splicing of the pre-mRNA variant occurs then the pre-mRNA variant is identical to the mRNA variant.

It is also known in the art that variants can be produced through the use of alternative signals to start or stop transcription and that pre-mRNAs and mRNAs can possess more that one start codon or stop codon. Variants that originate from a pre-mRNA or mRNA that use alternative start codons are known as "alternative start variants" of that pre-mRNA or mRNA. Those transcripts that use an alternative stop codon are known as "alternative stop variants" of that pre-mRNA or mRNA. One specific type of alternative stop variant is the "polyA variant" in which the multiple transcripts produced result from the alternative selection of one of the "polyA stop signals" by the transcription machinery, thereby producing transcripts that terminate at unique polyA sites. Within the context of the invention, the types of variants described herein are also preferred target nucleic acids.

The locations on the target nucleic acid to which the

preferred antisense compounds hybridize are hereinbelow referred to as "preferred target segments." As used herein the term "preferred target segment" is defined as at least an 8-nucleobase portion of a target region to which an active antisense compound is targeted. While not wishing to be bound by theory, it is presently believed that these target segments represent portions of the target nucleic acid which are accessible for hybridization.

While the specific sequences of certain preferred target segments are set forth herein, one of skill in the art will recognize that these serve to illustrate and describe particular embodiments within the scope of the present invention. Additional preferred target segments may be identified by one having ordinary skill.

Target segments 8-80 nucleobases in length comprising a stretch of at least eight (8) consecutive nucleobases selected from within the illustrative preferred target segments are considered to be suitable for targeting as well.

Target segments can include DNA or RNA sequences that comprise at least the 8 consecutive nucleobases from the 5'-terminus of one of the illustrative preferred target segments (the remaining nucleobases being a consecutive stretch of the same DNA or RNA beginning immediately upstream of the 5'-terminus of the target segment and continuing until the DNA or RNA contains about 8 to about 80 nucleobases). Similarly preferred target segments are represented by DNA or RNA sequences that comprise at least the 8 consecutive nucleobases from the 3'-terminus of one of the illustrative preferred target segments (the remaining nucleobases being a consecutive stretch of the same DNA or RNA beginning immediately downstream of the 3'-terminus of the target segment and continuing until the DNA or RNA contains about 8 to about 80 nucleobases). One having skill in the art armed

with the preferred target segments illustrated herein will be able, without undue experimentation, to identify further preferred target segments.

Once one or more target regions, segments or sites have been identified, antisense compounds are chosen which are sufficiently complementary to the target, i.e., hybridize sufficiently well and with sufficient specificity, to give the desired effect.

The oligomeric compounds are also targeted to or not targeted to regions of the target nucleobase sequence (e.g., such as those disclosed in Example 13) comprising nucleobases 1-50, 51-100, 101-150, 151-200, 201-250, 251-300, 301-350, 351-400, 401-450, 451-500, 501-550, 551-600, 601-650, 651-700, 701-750, 751-800, 801-850, 851-900, 901-950, 951-1000, 1001-1050, 1051-1100, 1101-1150, 1151-1200, 1201-1250, 1251-1300, 1301-1350, 1351-1400, 1401-1450, 1451-1500, 1501-1550, 1551-1600, 1601-1650, 1651-1700, 1701-1750, 1751-1800, 1801-1850, 1851-1900, 1901-1950, 1951-2000, 2001-2050, 2051-2100, 2101-2150, 2151-2200, 2201-2250, 2251-2300, 2301-2350, 2351-2400, 2401-2439, or any combination thereof.

D. Screening and Target Validation

In a further embodiment, the "preferred target segments" identified herein may be employed in a screen for additional compounds that modulate the expression of diacylglycerol acyltransferase 2. "Modulators" are those compounds that decrease or increase the expression of a nucleic acid molecule encoding diacylglycerol acyltransferase 2 and which comprise at least an 8-nucleobase portion which is complementary to a preferred target segment. The screening method comprises the steps of contacting a preferred target segment of a nucleic acid molecule encoding diacylglycerol acyltransferase 2 with one or more candidate

modulators, and selecting for one or more candidate modulators which decrease or increase the expression of a nucleic acid molecule encoding diacylglycerol acyltransferase 2. Once it is shown that the candidate modulator or modulators are capable of modulating (e.g. either decreasing or increasing) the expression of a nucleic acid molecule encoding diacylglycerol acyltransferase 2, the modulator may then be employed in further investigative studies of the function of diacylglycerol acyltransferase 2, or for use as a research, diagnostic, or therapeutic agent in accordance with the present invention.

The preferred target segments of the present invention may be also be combined with their respective complementary antisense compounds of the present invention to form stabilized double-stranded (duplexed) oligonucleotides.

Such double stranded oligonucleotide moieties have been shown in the art to modulate target expression and regulate translation as well as RNA processsing via an antisense mechanism. Moreover, the double-stranded moieties may be subject to chemical modifications (Fire et al., *Nature*, **1998**, *391*, 806-811; Timmons and Fire, *Nature* **1998**, *395*, 854; Timmons et al., *Gene*, **2001**, *263*, 103-112; Tabara et al., *Science,* **1998**, 282, 430-431; Montgomery et al., *Proc. Natl. Acad. Sci. USA*, **1998**, *95*, 15502-15507; Tuschl et al., *Genes Dev.*, **1999**, *13*, 3191-3197; Elbashir et al., *Nature*, **2001**, *411*, 494-498; Elbashir et al., *Genes Dev.* **2001**, *15*, 188-200). For example, such double-stranded moieties have been shown to inhibit the target by the classical hybridization of antisense strand of the duplex to the target, thereby triggering enzymatic degradation of the target (Tijsterman et al., *Science*, **2002**, *295*, 694-697).

The compounds of the present invention can also be applied in the areas of drug discovery and target validation.

The present invention comprehends the use of the compounds and preferred target segments identified herein in drug discovery efforts to elucidate relationships that exist between diacylglycerol acyltransferase 2 and a disease state, phenotype, or condition. These methods include detecting or modulating diacylglycerol acyltransferase 2 comprising contacting a sample, tissue, cell, or organism with the compounds of the present invention, measuring the nucleic acid or protein level of diacylglycerol acyltransferase 2 and/or a related phenotypic or chemical endpoint at some time after treatment, and optionally comparing the measured value to a non-treated sample or sample treated with a further compound of the invention. These methods can also be performed in parallel or in combination with other experiments to determine the function of unknown genes for the process of target validation or to determine the validity of a particular gene product as a target for treatment or prevention of a particular disease, condition, or phenotype.

E. Kits, Research Reagents, Diagnostics, and Therapeutics

The compounds of the present invention can be utilized for diagnostics, therapeutics, prophylaxis and as research reagents and kits. Furthermore, antisense oligonucleotides, which are able to inhibit gene expression with exquisite specificity, are often used by those of ordinary skill to elucidate the function of particular genes or to distinguish between functions of various members of a biological pathway.

For use in kits and diagnostics, the compounds of the present invention, either alone or in combination with other compounds or therapeutics, can be used as tools in differential and/or combinatorial analyses to elucidate expression patterns of a portion or the entire complement of genes expressed within cells and tissues.

As one nonlimiting example, expression patterns within cells or tissues treated with one or more antisense compounds are compared to control cells or tissues not treated with antisense compounds and the patterns produced are analyzed for differential levels of gene expression as they pertain, for example, to disease association, signaling pathway, cellular localization, expression level, size, structure or function of the genes examined. These analyses can be performed on stimulated or unstimulated cells and in the presence or absence of other compounds which affect expression patterns.

Examples of methods of gene expression analysis known in the art include DNA arrays or microarrays (Brazma and Vilo, *FEBS Lett.*, **2000,** 480, 17-24; Celis, *et al.*, *FEBS Lett.*, **2000,** 480, 2-16), SAGE (serial analysis of gene expression)(Madden, *et al.*, *Drug Discov. Today,* **2000,** 5, 415-425), READS (restriction enzyme amplification of digested cDNAs) (Prashar and Weissman, *Methods Enzymol.*, **1999,** 303, 258-72), TOGA (total gene expression analysis) (Sutcliffe, *et al.*, *Proc. Natl. Acad. Sci. U. S. A.*, **2000,** 97, 1976-81), protein arrays and proteomics (Celis, *et al.*, *FEBS Lett.*, **2000,** 480, 2-16; Jungblut, *et al.*, *Electrophoresis,* **1999,** 20, 2100-10), expressed sequence tag (EST) sequencing (Celis, *et al.*, *FEBS Lett.*, **2000,** 480, 2-16; Larsson, *et al.*, *J. Biotechnol.*, **2000,** 80, 143-57), subtractive RNA fingerprinting (SuRF) (Fuchs, *et al.*, *Anal. Biochem.*, **2000,** 286, 91-98; Larson, *et al.*, *Cytometry,* **2000,** 41, 203-208), subtractive cloning, differential display (DD) (Jurecic and Belmont, *Curr. Opin. Microbiol.*, **2000,** 3, 316-21), comparative genomic hybridization (Carulli, *et al.*, *J. Cell Biochem. Suppl.*, **1998,** 31, 286-96), FISH (fluorescent *in situ* hybridization) techniques (Going and Gusterson, *Eur. J. Cancer,* **1999,** 35, 1895-904) and mass spectrometry methods

(To, *Comb. Chem. High Throughput Screen*, **2000**, 3, 235-41).

The compounds of the invention are useful for research and diagnostics, because these compounds hybridize to nucleic acids encoding diacylglycerol acyltransferase 2. For example, oligonucleotides that are shown to hybridize with such efficiency and under such conditions as disclosed herein as to be effective diacylglycerol acyltransferase 2 inhibitors will also be effective primers or probes under conditions favoring gene amplification or detection, respectively. These primers and probes are useful in methods requiring the specific detection of nucleic acid molecules encoding diacylglycerol acyltransferase 2 and in the amplification of said nucleic acid molecules for detection or for use in further studies of diacylglycerol acyltransferase 2. Hybridization of the antisense oligonucleotides, particularly the primers and probes, of the invention with a nucleic acid encoding diacylglycerol acyltransferase 2 can be detected by means known in the art. Such means may include conjugation of an enzyme to the oligonucleotide, radiolabelling of the oligonucleotide or any other suitable detection means. Kits using such detection means for detecting the level of diacylglycerol acyltransferase 2 in a sample may also be prepared.

The specificity and sensitivity of antisense is also harnessed by those of skill in the art for therapeutic uses. Antisense compounds have been employed as therapeutic moieties in the treatment of disease states in animals, including humans. Antisense oligonucleotide drugs, including ribozymes, have been safely and effectively administered to humans and numerous clinical trials are presently underway. It is thus established that antisense compounds can be useful therapeutic modalities that can be configured to be useful in treatment regimes for the treatment of cells, tissues and

animals, especially humans.

For therapeutics, an animal, preferably a human, suspected of having a disease or disorder which can be treated by modulating the expression of diacylglycerol acyltransferase 2 is treated by administering antisense compounds in accordance with this invention. For example, in one non-limiting embodiment, the methods comprise the step of administering to the animal in need of treatment, a therapeutically effective amount of a diacylglycerol acyltransferase 2 inhibitor. The diacylglycerol acyltransferase 2 inhibitors of the present invention effectively inhibit the activity of the diacylglycerol acyltransferase 2 protein or inhibit the expression of the diacylglycerol acyltransferase 2 protein. In one embodiment, the activity or expression of diacylglycerol acyltransferase 2 in an animal is inhibited by about 10%. Preferably, the activity or expression of diacylglycerol acyltransferase 2 in an animal is inhibited by about 30%. More preferably, the activity or expression of diacylglycerol acyltransferase 2 in an animal is inhibited by 50% or more. Thus, the oligomeric compounds modulate expression of diacylglycerol acyltransferase 2 mRNA by at least 10%, by at least 20%, by at least 25%, by at least 30%, by at least 40%, by at least 50%, by at least 60%, by at least 70%, by at least 75%, by at least 80%, by at least 85%, by at least 90%, by at least 95%, by at least 98%, by at least 99%, or by 100%.

For example, the reduction of the expression of diacylglycerol acyltransferase 2 may be measured in serum, adipose tissue, liver or any other body fluid, tissue or organ of the animal. Preferably, the cells contained within said fluids, tissues or organs being analyzed contain a nucleic acid molecule encoding diacylglycerol acyltransferase

2 protein and/or the diacylglycerol acyltransferase 2 protein itself.

The compounds of the invention can be utilized in pharmaceutical compositions by adding an effective amount of a compound to a suitable pharmaceutically acceptable diluent or carrier. Use of the compounds and methods of the invention may also be useful prophylactically.

F. Modifications

As is known in the art, a nucleoside is a base-sugar combination. The base portion of the nucleoside is normally a heterocyclic base. The two most common classes of such heterocyclic bases are the purines and the pyrimidines. Nucleotides are nucleosides that further include a phosphate group covalently linked to the sugar portion of the nucleoside. For those nucleosides that include a pentofuranosyl sugar, the phosphate group can be linked to either the 2′, 3′ or 5′ hydroxyl moiety of the sugar. In forming oligonucleotides, the phosphate groups covalently link adjacent nucleosides to one another to form a linear polymeric compound. In turn, the respective ends of this linear polymeric compound can be further joined to form a circular compound, however, linear compounds are generally preferred. In addition, linear compounds may have internal nucleobase complementarity and may therefore fold in a manner as to produce a fully or partially double-stranded compound. Within oligonucleotides, the phosphate groups are commonly referred to as forming the internucleoside backbone of the oligonucleotide. The normal linkage or backbone of RNA and DNA is a 3′ to 5′ phosphodiester linkage.

Modified Internucleoside Linkages (Backbones)

Specific examples of preferred antisense compounds

useful in this invention include oligonucleotides containing modified backbones or non-natural internucleoside linkages. As defined in this specification, oligonucleotides having modified backbones include those that retain a phosphorus atom in the backbone and those that do not have a phosphorus atom in the backbone. For the purposes of this specification, and as sometimes referenced in the art, modified oligonucleotides that do not have a phosphorus atom in their internucleoside backbone can also be considered to be oligonucleosides.

Preferred modified oligonucleotide backbones containing a phosphorus atom therein include, for example, phosphorothioates, chiral phosphorothioates, phosphorodithioates, phosphotriesters, aminoalkylphosphotriesters, methyl and other alkyl phosphonates including 3'-alkylene phosphonates, 5'-alkylene phosphonates and chiral phosphonates, phosphinates, phosphoramidates including 3'-amino phosphoramidate and aminoalkylphosphoramidates, thionophosphoramidates, thionoalkylphosphonates, thionoalkylphosphotriesters, selenophosphates and boranophosphates having normal 3'-5' linkages, 2'-5' linked analogs of these, and those having inverted polarity wherein one or more internucleotide linkages is a 3' to 3', 5' to 5' or 2' to 2' linkage. Preferred oligonucleotides having inverted polarity comprise a single 3' to 3' linkage at the 3'-most internucleotide linkage i.e. a single inverted nucleoside residue which may be abasic (the nucleobase is missing or has a hydroxyl group in place thereof). Various salts, mixed salts and free acid forms are also included.

Representative United States patents that teach the preparation of the above phosphorus-containing linkages include, but are not limited to, U.S.: 3,687,808; 4,469,863; 4,476,301; 5,023,243; 5,177,196; 5,188,897; 5,264,423;

5,276,019; 5,278,302; 5,286,717; 5,321,131; 5,399,676; 5,405,939; 5,453,496; 5,455,233; 5,466,677; 5,476,925; 5,519,126; 5,536,821; 5,541,306; 5,550,111; 5,563,253; 5,571,799; 5,587,361; 5,194,599; 5,565,555; 5,527,899; 5,721,218; 5,672,697 and 5,625,050, certain of which are commonly owned with this application, and each of which is herein incorporated by reference.

Preferred modified oligonucleotide backbones that do not include a phosphorus atom therein have backbones that are formed by short chain alkyl or cycloalkyl internucleoside linkages, mixed heteroatom and alkyl or cycloalkyl internucleoside linkages, or one or more short chain heteroatomic or heterocyclic internucleoside linkages. These include those having morpholino linkages (formed in part from the sugar portion of a nucleoside); siloxane backbones; sulfide, sulfoxide and sulfone backbones; formacetyl and thioformacetyl backbones; methylene formacetyl and thioformacetyl backbones; riboacetyl backbones; alkene containing backbones; sulfamate backbones; methyleneimino and methylenehydrazino backbones; sulfonate and sulfonamide backbones; amide backbones; and others having mixed N, O, S and CH_2 component parts.

Representative United States patents that teach the preparation of the above oligonucleosides include, but are not limited to, U.S.: 5,034,506; 5,166,315; 5,185,444; 5,214,134; 5,216,141; 5,235,033; 5,264,562; 5,264,564; 5,405,938; 5,434,257; 5,466,677; 5,470,967; 5,489,677; 5,541,307; 5,561,225; 5,596,086; 5,602,240; 5,610,289; 5,602,240; 5,608,046; 5,610,289; 5,618,704; 5,623,070; 5,663,312; 5,633,360; 5,677,437; 5,792,608; 5,646,269 and 5,677,439, certain of which are commonly owned with this application, and each of which is herein incorporated by reference.

Modified sugar and internucleoside linkages-Mimetics

In other preferred oligonucleotide mimetics, both the sugar and the internucleoside linkage (i.e. the backbone), of the nucleotide units are replaced with novel groups. The nucleobase units are maintained for hybridization with an appropriate target nucleic acid. One such compound, an oligonucleotide mimetic that has been shown to have excellent hybridization properties, is referred to as a peptide nucleic acid (PNA). In PNA compounds, the sugar-backbone of an oligonucleotide is replaced with an amide containing backbone, in particular an aminoethylglycine backbone. The nucleobases are retained and are bound directly or indirectly to aza nitrogen atoms of the amide portion of the backbone. Representative United States patents that teach the preparation of PNA compounds include, but are not limited to, U.S.: 5,539,082; 5,714,331; and 5,719,262, each of which is herein incorporated by reference. Further teaching of PNA compounds can be found in Nielsen *et al.*, *Science*, **1991**, 254, 1497-1500.

Preferred embodiments of the invention are oligonucleotides with phosphorothioate backbones and oligonucleosides with heteroatom backbones, and in particular $-CH_2-NH-O-CH_2-$, $-CH_2-N(CH_3)-O-CH_2-$ [known as a methylene (methylimino) or MMI backbone], $-CH_2-O-N(CH_3)-CH_2-$, $-CH_2-N(CH_3)-N(CH_3)-CH_2-$ and $-O-N(CH_3)-CH_2-CH_2-$ [wherein the native phosphodiester backbone is represented as $-O-P-O-CH_2-$] of the above referenced U.S. patent 5,489,677, and the amide backbones of the above referenced U.S. patent 5,602,240. Also preferred are oligonucleotides having morpholino backbone structures of the above-referenced U.S. patent 5,034,506.

Modified sugars

Modified oligonucleotides may also contain one or more substituted sugar moieties. Preferred oligonucleotides comprise one of the following at the 2' position: OH; F; O-, S-, or N-alkyl; O-, S-, or N-alkenyl; O-, S- or N-alkynyl; or O-alkyl-O-alkyl, wherein the alkyl, alkenyl and alkynyl may be substituted or unsubstituted C_1 to C_{10} alkyl or C_2 to C_{10} alkenyl and alkynyl. Particularly preferred are $O[(CH_2)_nO]_mCH_3$, $O(CH_2)_nOCH_3$, $O(CH_2)_nNH_2$, $O(CH_2)_nCH_3$, $O(CH_2)_nONH_2$, and $O(CH_2)_nON[(CH_2)_nCH_3]_2$, where n and m are from 1 to about 10. Other preferred oligonucleotides comprise one of the following at the 2' position: C_1 to C_{10} lower alkyl, substituted lower alkyl, alkenyl, alkynyl, alkaryl, aralkyl, O-alkaryl or O-aralkyl, SH, SCH_3, OCN, Cl, Br, CN, CF_3, OCF_3, $SOCH_3$, SO_2CH_3, ONO_2, NO_2, N_3, NH_2, heterocycloalkyl, heterocycloalkaryl, aminoalkylamino, polyalkylamino, substituted silyl, an RNA cleaving group, a reporter group, an intercalator, a group for improving the pharmacokinetic properties of an oligonucleotide, or a group for improving the pharmacodynamic properties of an oligonucleotide, and other substituents having similar properties. A preferred modification includes 2'-methoxyethoxy (2'-O-$CH_2CH_2OCH_3$, also known as 2'-O-(2-methoxyethyl) or 2'-MOE) (Martin *et al.*, *Helv. Chim. Acta*, **1995**, *78*, 486-504) i.e., an alkoxyalkoxy group. A further preferred modification includes 2'-dimethylaminooxyethoxy, i.e., a $O(CH_2)_2ON(CH_3)_2$ group, also known as 2'-DMAOE, as described in examples hereinbelow, and 2'-dimethylaminoethoxyethoxy (also known in the art as 2'-O-dimethyl-amino-ethoxy-ethyl or 2'-DMAEOE), i.e., 2'-O-CH_2-O-CH_2-$N(CH_3)_2$, also described in examples hereinbelow.

Other preferred modifications include 2'-methoxy (2'-O-CH_3), 2'-aminopropoxy (2'-$OCH_2CH_2CH_2NH_2$), 2'-allyl (2'-CH_2-$CH=CH_2$), 2'-O-allyl (2'-O-CH_2-$CH=CH_2$) and 2'-fluoro (2'-F).

The 2'-modification may be in the arabino (up) position or ribo (down) position. A preferred 2'-arabino modification is 2'-F. Similar modifications may also be made at other positions on the oligonucleotide, particularly the 3' position of the sugar on the 3' terminal nucleotide or in 2'-5' linked oligonucleotides and the 5' position of 5' terminal nucleotide. Oligonucleotides may also have sugar mimetics such as cyclobutyl moieties in place of the pentofuranosyl sugar. Representative United States patents that teach the preparation of such modified sugar structures include, but are not limited to, U.S.: 4,981,957; 5,118,800; 5,319,080; 5,359,044; 5,393,878; 5,446,137; 5,466,786; 5,514,785; 5,519,134; 5,567,811; 5,576,427; 5,591,722; 5,597,909; 5,610,300; 5,627,053; 5,639,873; 5,646,265; 5,658,873; 5,670,633; 5,792,747; and 5,700,920, certain of which are commonly owned with the instant application, and each of which is herein incorporated by reference in its entirety.

A further preferred modification of the sugar includes Locked Nucleic Acids (LNAs) in which the 2'-hydroxyl group is linked to the 3' or 4' carbon atom of the sugar ring, thereby forming a bicyclic sugar moiety. The linkage is preferably a methylene $(-CH_2-)_n$ group bridging the 2' oxygen atom and the 4' carbon atom wherein n is 1 or 2. LNAs and preparation thereof are described in WO 98/39352 and WO 99/14226.

Natural and Modified Nucleobases

Oligonucleotides may also include nucleobase (often referred to in the art simply as "base") modifications or substitutions. As used herein, "unmodified" or "natural" nucleobases include the purine bases adenine (A) and guanine (G), and the pyrimidine bases thymine (T), cytosine (C) and uracil (U). Modified nucleobases include other synthetic and natural nucleobases such as 5-methylcytosine (5-me-C), 5-

hydroxymethyl cytosine, xanthine, hypoxanthine, 2-aminoadenine, 6-methyl and other alkyl derivatives of adenine and guanine, 2-propyl and other alkyl derivatives of adenine and guanine, 2-thiouracil, 2-thiothymine and 2-thiocytosine, 5-halouracil and cytosine, 5-propynyl (-C?C-CH_3) uracil and cytosine and other alkynyl derivatives of pyrimidine bases, 6-azo uracil, cytosine and thymine, 5-uracil (pseudouracil), 4-thiouracil, 8-halo, 8-amino, 8-thiol, 8-thioalkyl, 8-hydroxyl and other 8-substituted adenines and guanines, 5-halo particularly 5-bromo, 5-trifluoromethyl and other 5-substituted uracils and cytosines, 7-methylguanine and 7-methyladenine, 2-F-adenine, 2-amino-adenine, 8-azaguanine and 8-azaadenine, 7-deazaguanine and 7-deazaadenine and 3-deazaguanine and 3-deazaadenine. Further modified nucleobases include tricyclic pyrimidines such as phenoxazine cytidine(1H-pyrimido[5,4-b][1,4]benzoxazin-2(3H)-one), phenothiazine cytidine (1H-pyrimido[5,4-b][1,4]benzothiazin-2(3H)-one), G-clamps such as a substituted phenoxazine cytidine (e.g. 9-(2-aminoethoxy)-H-pyrimido[5,4-b][1,4]benzoxazin-2(3H)-one), carbazole cytidine (2H-pyrimido[4,5-b]indol-2-one), pyridoindole cytidine (H-pyrido[3',2':4,5]pyrrolo[2,3-d]pyrimidin-2-one). Modified nucleobases may also include those in which the purine or pyrimidine base is replaced with other heterocycles, for example 7-deaza-adenine, 7-deazaguanosine, 2-aminopyridine and 2-pyridone. Further nucleobases include those disclosed in United States Patent No. 3,687,808, those disclosed in *The Concise Encyclopedia Of Polymer Science And Engineering*, pages 858-859, Kroschwitz, J.I., ed. John Wiley & Sons, **1990**, those disclosed by Englisch *et al.*, *Angewandte Chemie*, International Edition, **1991**, *30*, 613, and those disclosed by Sanghvi, Y.S., Chapter 15, *Antisense Research and Applications*, pages 289-302, Crooke, S.T. and Lebleu, B. ,

ed., CRC Press, **1993**. Certain of these nucleobases are particularly useful for increasing the binding affinity of the compounds of the invention. These include 5-substituted pyrimidines, 6-azapyrimidines and N-2, N-6 and O-6 substituted purines, including 2-aminopropyladenine, 5-propynyluracil and 5-propynylcytosine. 5-methylcytosine substitutions have been shown to increase nucleic acid duplex stability by 0.6-1.2 ?C and are presently preferred base substitutions, even more particularly when combined with 2'-O-methoxyethyl sugar modifications.

Representative United States patents that teach the preparation of certain of the above noted modified nucleobases as well as other modified nucleobases include, but are not limited to, the above noted U.S. 3,687,808, as well as U.S.: 4,845,205; 5,130,302; 5,134,066; 5,175,273; 5,367,066; 5,432,272; 5,457,187; 5,459,255; 5,484,908; 5,502,177; 5,525,711; 5,552,540; 5,587,469; 5,594,121, 5,596,091; 5,614,617; 5,645,985; 5,830,653; 5,763,588; 6,005,096; and 5,681,941, certain of which are commonly owned with the instant application, and each of which is herein incorporated by reference, and United States patent 5,750,692, which is commonly owned with the instant application and also herein incorporated by reference.

Conjugates

Another modification of the oligonucleotides of the invention involves chemically linking to the oligonucleotide one or more moieties or conjugates which enhance the activity, cellular distribution or cellular uptake of the oligonucleotide. These moieties or conjugates can include conjugate groups covalently bound to functional groups such as primary or secondary hydroxyl groups. Conjugate groups of the invention include intercalators, reporter molecules,

polyamines, polyamides, polyethylene glycols, polyethers, groups that enhance the pharmacodynamic properties of oligomers, and groups that enhance the pharmacokinetic properties of oligomers. Typical conjugate groups include cholesterols, lipids, phospholipids, biotin, phenazine, folate, phenanthridine, anthraquinone, acridine, fluoresceins, rhodamines, coumarins, and dyes. Groups that enhance the pharmacodynamic properties, in the context of this invention, include groups that improve uptake, enhance resistance to degradation, and/or strengthen sequence-specific hybridization with the target nucleic acid. Groups that enhance the pharmacokinetic properties, in the context of this invention, include groups that improve uptake, distribution, metabolism or excretion of the compounds of the present invention. Representative conjugate groups are disclosed in International Patent Application PCT/US92/09196, filed October 23, 1992, and U.S. Patent 6,287,860, the entire disclosure of which are incorporated herein by reference. Conjugate moieties include but are not limited to lipid moieties such as a cholesterol moiety, cholic acid, a thioether, e.g., hexyl-S-tritylthiol, a thiocholesterol, an aliphatic chain, e.g., dodecandiol or undecyl residues, a phospholipid, e.g., di-hexadecyl-rac-glycerol or triethylammonium 1,2-di-O-hexadecyl-rac-glycero-3-H-phosphonate, a polyamine or a polyethylene glycol chain, or adamantane acetic acid, a palmityl moiety, or an octadecylamine or hexylamino-carbonyl-oxycholesterol moiety. Oligonucleotides of the invention may also be conjugated to active drug substances, for example, aspirin, warfarin, phenylbutazone, ibuprofen, suprofen, fenbufen, ketoprofen, (*S*)-(+)-pranoprofen, carprofen, dansylsarcosine, 2,3,5-triiodobenzoic acid, flufenamic acid, folinic acid, a benzothiadiazide, chlorothiazide, a diazepine, indomethicin, a barbiturate, a

cephalosporin, a sulfa drug, an antidiabetic, an antibacterial or an antibiotic. Oligonucleotide-drug conjugates and their preparation are described in United States Patent Application 09/334,130 (filed June 15, 1999) which is incorporated herein by reference in its entirety.

Representative United States patents that teach the preparation of such oligonucleotide conjugates include, but are not limited to, U.S.: 4,828,979; 4,948,882; 5,218,105; 5,525,465; 5,541,313; 5,545,730; 5,552,538; 5,578,717, 5,580,731; 5,580,731; 5,591,584; 5,109,124; 5,118,802; 5,138,045; 5,414,077; 5,486,603; 5,512,439; 5,578,718; 5,608,046; 4,587,044; 4,605,735; 4,667,025; 4,762,779; 4,789,737; 4,824,941; 4,835,263; 4,876,335; 4,904,582; 4,958,013; 5,082,830; 5,112,963; 5,214,136; 5,082,830; 5,112,963; 5,214,136; 5,245,022; 5,254,469; 5,258,506; 5,262,536; 5,272,250; 5,292,873; 5,317,098; 5,371,241, 5,391,723; 5,416,203, 5,451,463; 5,510,475; 5,512,667; 5,514,785; 5,565,552; 5,567,810; 5,574,142; 5,585,481; 5,587,371; 5,595,726; 5,597,696; 5,599,923; 5,599,928 and 5,688,941, certain of which are commonly owned with the instant application, and each of which is herein incorporated by reference.

Chimeric compounds

It is not necessary for all positions in a given compound to be uniformly modified, and in fact more than one of the aforementioned modifications may be incorporated in a single compound or even at a single nucleoside within an oligonucleotide.

The present invention also includes antisense compounds which are chimeric compounds. "Chimeric" antisense compounds or "chimeras," in the context of this invention, are antisense compounds, particularly oligonucleotides, which

contain two or more chemically distinct regions, each made up of at least one monomer unit, i.e., a nucleotide in the case of an oligonucleotide compound. These oligonucleotides typically contain at least one region wherein the oligonucleotide is modified so as to confer upon the oligonucleotide increased resistance to nuclease degradation, increased cellular uptake, increased stability and/or increased binding affinity for the target nucleic acid. An additional region of the oligonucleotide may serve as a substrate for enzymes capable of cleaving RNA:DNA or RNA:RNA hybrids. By way of example, RNAse H is a cellular endonuclease which cleaves the RNA strand of an RNA:DNA duplex. Activation of RNase H, therefore, results in cleavage of the RNA target, thereby greatly enhancing the efficiency of oligonucleotide-mediated inhibition of gene expression. The cleavage of RNA:RNA hybrids can, in like fashion, be accomplished through the actions of endoribonucleases, such as RNAseL which cleaves both cellular and viral RNA. Cleavage of the RNA target can be routinely detected by gel electrophoresis and, if necessary, associated nucleic acid hybridization techniques known in the art.

Chimeric antisense compounds of the invention may be formed as composite structures of two or more oligonucleotides, modified oligonucleotides, oligonucleosides and/or oligonucleotide mimetics as described above. Such compounds have also been referred to in the art as hybrids or gapmers. Representative United States patents that teach the preparation of such hybrid structures include, but are not limited to, U.S.: 5,013,830; 5,149,797; 5,220,007; 5,256,775; 5,366,878; 5,403,711; 5,491,133; 5,565,350; 5,623,065; 5,652,355; 5,652,356; and 5,700,922, certain of which are commonly owned with the instant application, and each of which is herein incorporated by reference in its entirety.

G. Formulations

The compounds of the invention may also be admixed, encapsulated, conjugated or otherwise associated with other molecules, molecule structures or mixtures of compounds, as for example, liposomes, receptor-targeted molecules, oral, rectal, topical or other formulations, for assisting in uptake, distribution and/or absorption. Representative United States patents that teach the preparation of such uptake, distribution and/or absorption-assisting formulations include, but are not limited to, U.S.: 5,108,921; 5,354,844; 5,416,016; 5,459,127; 5,521,291; 5,543,158; 5,547,932; 5,583,020; 5,591,721; 4,426,330; 4,534,899; 5,013,556; 5,108,921; 5,213,804; 5,227,170; 5,264,221; 5,356,633; 5,395,619; 5,416,016; 5,417,978; 5,462,854; 5,469,854; 5,512,295; 5,527,528; 5,534,259; 5,543,152; 5,556,948; 5,580,575; and 5,595,756, each of which is herein incorporated by reference.

The antisense compounds of the invention encompass any pharmaceutically acceptable salts, esters, or salts of such esters, or any other compound which, upon administration to an animal, including a human, is capable of providing (directly or indirectly) the biologically active metabolite or residue thereof.

The term "pharmaceutically acceptable salts" refers to physiologically and pharmaceutically acceptable salts of the compounds of the invention: i.e., salts that retain the desired biological activity of the parent compound and do not impart undesired toxicological effects thereto. For oligonucleotides, preferred examples of pharmaceutically acceptable salts and their uses are further described in U.S. Patent 6,287,860, which is incorporated herein in its entirety.

The present invention also includes pharmaceutical compositions and formulations which include the antisense compounds of the invention. The pharmaceutical compositions of the present invention may be administered in a number of ways depending upon whether local or systemic treatment is desired and upon the area to be treated. Administration may be topical (including ophthalmic and to mucous membranes including vaginal and rectal delivery), pulmonary, e.g., by inhalation or insufflation of powders or aerosols, including by nebulizer; intratracheal, intranasal, epidermal and transdermal), oral or parenteral. Parenteral administration includes intravenous, intraarterial, subcutaneous, intraperitoneal or intramuscular injection or infusion; or intracranial, e.g., intrathecal or intraventricular, administration. Oligonucleotides with at least one 2'-O-methoxyethyl modification are believed to be particularly useful for oral administration. Pharmaceutical compositions and formulations for topical administration may include transdermal patches, ointments, lotions, creams, gels, drops, suppositories, sprays, liquids and powders. Conventional pharmaceutical carriers, aqueous, powder or oily bases, thickeners and the like may be necessary or desirable. Coated condoms, gloves and the like may also be useful.

The pharmaceutical formulations of the present invention, which may conveniently be presented in unit dosage form, may be prepared according to conventional techniques well known in the pharmaceutical industry. Such techniques include the step of bringing into association the active ingredients with the pharmaceutical carrier(s) or excipient(s). In general, the formulations are prepared by uniformly and intimately bringing into association the active ingredients with liquid carriers or finely divided solid carriers or both, and then, if necessary, shaping the

product.

The compositions of the present invention may be formulated into any of many possible dosage forms such as, but not limited to, tablets, capsules, gel capsules, liquid syrups, soft gels, suppositories, and enemas. The compositions of the present invention may also be formulated as suspensions in aqueous, non-aqueous or mixed media. Aqueous suspensions may further contain substances which increase the viscosity of the suspension including, for example, sodium carboxymethylcellulose, sorbitol and/or dextran. The suspension may also contain stabilizers.

Pharmaceutical compositions of the present invention include, but are not limited to, solutions, emulsions, foams and liposome-containing formulations. The pharmaceutical compositions and formulations of the present invention may comprise one or more penetration enhancers, carriers, excipients or other active or inactive ingredients.

Emulsions are typically heterogenous systems of one liquid dispersed in another in the form of droplets usually exceeding 0.1 ?m in diameter. Emulsions may contain additional components in addition to the dispersed phases, and the active drug which may be present as a solution in either the aqueous phase, oily phase or itself as a separate phase. Microemulsions are included as an embodiment of the present invention. Emulsions and their uses are well known in the art and are further described in U.S. Patent 6,287,860, which is incorporated herein in its entirety.

Formulations of the present invention include liposomal formulations. As used in the present invention, the term "liposome" means a vesicle composed of amphiphilic lipids arranged in a spherical bilayer or bilayers. Liposomes are unilamellar or multilamellar vesicles which have a membrane formed from a lipophilic material and an aqueous interior

that contains the composition to be delivered. Cationic liposomes are positively charged liposomes which are believed to interact with negatively charged DNA molecules to form a stable complex. Liposomes that are pH-sensitive or negatively-charged are believed to entrap DNA rather than complex with it. Both cationic and noncationic liposomes have been used to deliver DNA to cells.

Liposomes also include "sterically stabilized" liposomes, a term which, as used herein, refers to liposomes comprising one or more specialized lipids that, when incorporated into liposomes, result in enhanced circulation lifetimes relative to liposomes lacking such specialized lipids. Examples of sterically stabilized liposomes are those in which part of the vesicle-forming lipid portion of the liposome comprises one or more glycolipids or is derivatized with one or more hydrophilic polymers, such as a polyethylene glycol (PEG) moiety. Liposomes and their uses are further described in U.S. Patent 6,287,860, which is incorporated herein in its entirety.

The pharmaceutical formulations and compositions of the present invention may also include surfactants. The use of surfactants in drug products, formulations and in emulsions is well known in the art. Surfactants and their uses are further described in U.S. Patent 6,287,860, which is incorporated herein in its entirety.

In one embodiment, the present invention employs various penetration enhancers to effect the efficient delivery of nucleic acids, particularly oligonucleotides. In addition to aiding the diffusion of non-lipophilic drugs across cell membranes, penetration enhancers also enhance the permeability of lipophilic drugs. Penetration enhancers may be classified as belonging to one of five broad categories, *i.e.*, surfactants, fatty acids, bile salts, chelating agents,

and non-chelating non-surfactants. Penetration enhancers and their uses are further described in U.S. Patent 6,287,860, which is incorporated herein in its entirety.

One of skill in the art will recognize that formulations are routinely designed according to their intended use, i.e. route of administration.

Preferred formulations for topical administration include those in which the oligonucleotides of the invention are in admixture with a topical delivery agent such as lipids, liposomes, fatty acids, fatty acid esters, steroids, chelating agents and surfactants. Preferred lipids and liposomes include neutral (e.g. dioleoylphosphatidyl DOPE ethanolamine, dimyristoylphosphatidyl choline DMPC, distearolyphosphatidyl choline) negative (e.g. dimyristoylphosphatidyl glycerol DMPG) and cationic (e.g. dioleoyltetramethylaminopropyl DOTAP and dioleoylphosphatidyl ethanolamine DOTMA).

For topical or other administration, oligonucleotides of the invention may be encapsulated within liposomes or may form complexes thereto, in particular to cationic liposomes. Alternatively, oligonucleotides may be complexed to lipids, in particular to cationic lipids. Preferred fatty acids and esters, pharmaceutically acceptable salts thereof, and their uses are further described in U.S. Patent 6,287,860, which is incorporated herein in its entirety. Topical formulations are described in detail in United States patent application 09/315,298 filed on May 20, 1999, which is incorporated herein by reference in its entirety.

Compositions and formulations for oral administration include powders or granules, microparticulates, nanoparticulates, suspensions or solutions in water or non-aqueous media, capsules, gel capsules, sachets, tablets or minitablets. Thickeners, flavoring agents, diluents,

emulsifiers, dispersing aids or binders may be desirable. Preferred oral formulations are those in which oligonucleotides of the invention are administered in conjunction with one or more penetration enhancers surfactants and chelators. Preferred surfactants include fatty acids and/or esters or salts thereof, bile acids and/or salts thereof. Preferred bile acids/salts and fatty acids and their uses are further described in U.S. Patent 6,287,860, which is incorporated herein in its entirety. Also preferred are combinations of penetration enhancers, for example, fatty acids/salts in combination with bile acids/salts. A particularly preferred combination is the sodium salt of lauric acid, capric acid and UDCA. Further penetration enhancers include polyoxyethylene-9-lauryl ether, polyoxyethylene-20-cetyl ether. Oligonucleotides of the invention may be delivered orally, in granular form including sprayed dried particles, or complexed to form micro or nanoparticles. Oligonucleotide complexing agents and their uses are further described in U.S. Patent 6,287,860, which is incorporated herein in its entirety. Oral formulations for oligonucleotides and their preparation are described in detail in United States applications 09/108,673 (filed July 1, 1998), 09/315,298 (filed May 20, 1999) and 10/071,822, filed February 8, 2002, each of which is incorporated herein by reference in their entirety.

Compositions and formulations for parenteral, intrathecal or intraventricular administration may include sterile aqueous solutions which may also contain buffers, diluents and other suitable additives such as, but not limited to, penetration enhancers, carrier compounds and other pharmaceutically acceptable carriers or excipients.

Certain embodiments of the invention provide pharmaceutical compositions containing one or more oligomeric

compounds and one or more other chemotherapeutic agents which function by a non-antisense mechanism. Examples of such chemotherapeutic agents include but are not limited to cancer chemotherapeutic drugs such as daunorubicin, daunomycin, dactinomycin, doxorubicin, epirubicin, idarubicin, esorubicin, bleomycin, mafosfamide, ifosfamide, cytosine arabinoside, bis-chloroethylnitrosurea, busulfan, mitomycin C, actinomycin D, mithramycin, prednisone, hydroxyprogesterone, testosterone, tamoxifen, dacarbazine, procarbazine, hexamethylmelamine, pentamethylmelamine, mitoxantrone, amsacrine, chlorambucil, methylcyclohexylnitrosurea, nitrogen mustards, melphalan, cyclophosphamide, 6-mercaptopurine, 6-thioguanine, cytarabine, 5-azacytidine, hydroxyurea, deoxycoformycin, 4-hydroxyperoxycyclophosphoramide, 5-fluorouracil (5-FU), 5-fluorodeoxyuridine (5-FUdR), methotrexate (MTX), colchicine, taxol, vincristine, vinblastine, etoposide (VP-16), trimetrexate, irinotecan, topotecan, gemcitabine, teniposide, cisplatin and diethylstilbestrol (DES). When used with the compounds of the invention, such chemotherapeutic agents may be used individually (*e.g.*, 5-FU and oligonucleotide), sequentially (*e.g.*, 5-FU and oligonucleotide for a period of time followed by MTX and oligonucleotide), or in combination with one or more other such chemotherapeutic agents (*e.g.*, 5-FU, MTX and oligonucleotide, or 5-FU, radiotherapy and oligonucleotide). Anti-inflammatory drugs, including but not limited to nonsteroidal anti-inflammatory drugs and corticosteroids, and antiviral drugs, including but not limited to ribivirin, vidarabine, acyclovir and ganciclovir, may also be combined in compositions of the invention. Combinations of antisense compounds and other non-antisense drugs are also within the scope of this invention. Two or more combined compounds may be used together or sequentially.

In another related embodiment, compositions of the invention may contain one or more antisense compounds, particularly oligonucleotides, targeted to a first nucleic acid and one or more additional antisense compounds targeted to a second nucleic acid target. Alternatively, compositions of the invention may contain two or more antisense compounds targeted to different regions of the same nucleic acid target. Numerous examples of antisense compounds are known in the art. Two or more combined compounds may be used together or sequentially.

H. Dosing

The formulation of therapeutic compositions and their subsequent administration (dosing) is believed to be within the skill of those in the art. Dosing is dependent on severity and responsiveness of the disease state to be treated, with the course of treatment lasting from several days to several months, or until a cure is effected or a diminution of the disease state is achieved. Optimal dosing schedules can be calculated from measurements of drug accumulation in the body of the patient. Persons of ordinary skill can easily determine optimum dosages, dosing methodologies and repetition rates. Optimum dosages may vary depending on the relative potency of individual oligonucleotides, and can generally be estimated based on EC_{50}s found to be effective in *in vitro* and *in vivo* animal models. In general, dosage is from 0.01 ug to 100 g per kg of body weight, and may be given once or more daily, weekly, monthly or yearly, or even once every 2 to 20 years. Persons of ordinary skill in the art can easily estimate repetition rates for dosing based on measured residence times and concentrations of the drug in bodily fluids or tissues. Following successful treatment, it may be desirable to have

the patient undergo maintenance therapy to prevent the recurrence of the disease state, wherein the oligonucleotide is administered in maintenance doses, ranging from 0.01 ug to 100 g per kg of body weight, once or more daily, to once every 20 years.

While the present invention has been described with specificity in accordance with certain of its preferred embodiments, the following examples serve only to illustrate the invention and are not intended to limit the same. Each of the references, GenBank accession numbers, and the like recited in the present application is incorporated herein by reference in its entirety.

EXAMPLES

Example 1

Synthesis of Nucleoside Phosphoramidites

The following compounds, including amidites and their intermediates were prepared as described in US Patent 6,426,220 and published PCT WO 02/36743; 5'-O-Dimethoxytrityl-thymidine intermediate for 5-methyl dC amidite, 5'-O-Dimethoxytrityl-2'-deoxy-5-methylcytidine intermediate for 5-methyl-dC amidite, 5'-O-Dimethoxytrityl-2'-deoxy-N4-benzoyl-5-methylcytidine penultimate intermediate for 5-methyl dC amidite, [5'-*O*-(4,4'-Dimethoxytriphenylmethyl)-2'-deoxy-N^4-benzoyl-5-methylcytidin-3'-*O*-yl]-2-cyanoethyl-*N,N*-diisopropylphosphoramidite (5-methyl dC amidite), 2'-Fluorodeoxyadenosine, 2'-Fluorodeoxyguanosine, 2'-Fluorouridine, 2'-Fluorodeoxycytidine, 2'-O-(2-Methoxyethyl) modified amidites, 2'-O-(2-methoxyethyl)-5-methyluridine intermediate, 5'-O-DMT-2'-O-(2-methoxyethyl)-5-methyluridine penultimate intermediate, [5'-*O*-(4,4'-Dimethoxytriphenylmethyl)-2'-*O*-(2-methoxyethyl)-5-methyluridin-3'-*O*-yl]-2-cyanoethyl-*N,N*-diisopropylphosphoramidite (MOE T amidite), 5'-O-Dimethoxytrityl-2'-O-(2-methoxyethyl)-5-methylcytidine intermediate, 5'-O-dimethoxytrityl-2'-*O*-(2-methoxyethyl)-N^4-benzoyl-5-methyl-cytidine penultimate intermediate, [5'-*O*-(4,4'-Dimethoxytriphenylmethyl)-2'-*O*-(2-methoxyethyl)-N^4-benzoyl-5-methylcytidin-3'-*O*-yl]-2-cyanoethyl-*N,N*-diisopropylphosphoramidite (MOE 5-Me-C amidite), [5'-*O*-(4,4'-Dimethoxytriphenylmethyl)-2'-*O*-(2-methoxyethyl)-N^6-benzoyladenosin-3'-*O*-yl]-2-cyanoethyl-*N,N*-diisopropylphosphoramidite (MOE A amdite), [5'-*O*-(4,4'-Dimethoxytriphenylmethyl)-2'-*O*-(2-methoxyethyl)-N^4-

isobutyrylguanosin-3'-*O*-yl]-2-cyanoethyl-*N,N*-diisopropylphosphoramidite (MOE G amidite), 2'-O-(Aminooxyethyl) nucleoside amidites and 2'-O-(dimethylamino-oxyethyl) nucleoside amidites, 2'-(Dimethylaminooxyethoxy) nucleoside amidites, 5'-O-tert-Butyldiphenylsilyl-O^2-2'-anhydro-5-methyluridine , 5'-O-tert-Butyldiphenylsilyl-2'-O-(2-hydroxyethyl)-5-methyluridine, 2'-O-([2-phthalimidoxy)ethyl]-5'-*t*-butyldiphenylsilyl-5-methyluridine , 5'-O-*tert*-butyldiphenylsilyl-2'-O-[(2-formadoximinooxy)ethyl]-5-methyluridine, 5'-O-*tert*-Butyldiphenylsilyl-2'-O-[N,N dimethylaminooxyethyl]-5-methyluridine, 2'-O-(dimethylaminooxyethyl)-5-methyluridine, 5'-O-DMT-2'-O-(dimethylaminooxyethyl)-5-methyluridine, 5'-O-DMT-2'-O-(2-N,N-dimethylaminooxyethyl)-5-methyluridine-3'-[(2-cyanoethyl)-N,N-diisopropylphosphoramidite], 2'-(Aminooxyethoxy) nucleoside amidites, N2-isobutyryl-6-O-diphenylcarbamoyl-2'-O-(2-ethylacetyl)-5'-O-(4,4'-dimethoxytrityl)guanosine-3'-[(2-cyanoethyl)-N,N-diisopropylphosphoramidite], 2'-dimethylaminoethoxyethoxy (2'-DMAEOE) nucleoside amidites, 2'-O-[2(2-N,N-dimethylaminoethoxy)ethyl]-5-methyl uridine, 5'-O-dimethoxytrityl-2'-O-[2(2-N,N-dimethylaminoethoxy)-ethyl)]-5-methyl uridine and 5'-O-Dimethoxytrityl-2'-O-[2(2-N,N-dimethylaminoethoxy)-ethyl)]-5-methyl uridine-3'-O-(cyanoethyl-N,N-diisopropyl)phosphoramidite.

Example 2

Oligonucleotide and oligonucleoside synthesis

The antisense compounds used in accordance with this invention may be conveniently and routinely made through the well-known technique of solid phase synthesis. Equipment for such synthesis is sold by several vendors including, for example, Applied Biosystems (Foster City, CA). Any other

means for such synthesis known in the art may additionally or alternatively be employed. It is well known to use similar techniques to prepare oligonucleotides such as the phosphorothioates and alkylated derivatives.

Oligonucleotides: Unsubstituted and substituted phosphodiester (P=O) oligonucleotides are synthesized on an automated DNA synthesizer (Applied Biosystems model 394) using standard phosphoramidite chemistry with oxidation by iodine.

Phosphorothioates (P=S) are synthesized similar to phosphodiester oligonucleotides with the following exceptions: thiation was effected by utilizing a 10% w/v solution of 3,H-1,2-benzodithiole-3-one 1,1-dioxide in acetonitrile for the oxidation of the phosphite linkages. The thiation reaction step time was increased to 180 sec and preceded by the normal capping step. After cleavage from the CPG column and deblocking in concentrated ammonium hydroxide at 55?C (12-16 hr), the oligonucleotides were recovered by precipitating with >3 volumes of ethanol from a 1 M NH_4OAc solution. Phosphinate oligonucleotides are prepared as described in U.S. Patent 5,508,270, herein incorporated by reference.

Alkyl phosphonate oligonucleotides are prepared as described in U.S. Patent 4,469,863, herein incorporated by reference.

3'-Deoxy-3'-methylene phosphonate oligonucleotides are prepared as described in U.S. Patents 5,610,289 or 5,625,050, herein incorporated by reference.

Phosphoramidite oligonucleotides are prepared as described in U.S. Patent, 5,256,775 or U.S. Patent 5,366,878, herein incorporated by reference.

Alkylphosphonothioate oligonucleotides are prepared as

described in published PCT applications PCT/US94/00902 and PCT/US93/06976 (published as WO 94/17093 and WO 94/02499, respectively), herein incorporated by reference.

3'-Deoxy-3'-amino phosphoramidate oligonucleotides are prepared as described in U.S. Patent 5,476,925, herein incorporated by reference.

Phosphotriester oligonucleotides are prepared as described in U.S. Patent 5,023,243, herein incorporated by reference.

Borano phosphate oligonucleotides are prepared as described in U.S. Patents 5,130,302 and 5,177,198, both herein incorporated by reference.

Oligonucleosides: Methylenemethylimino linked oligonucleosides, also identified as MMI linked oligonucleosides, methylenedimethylhydrazo linked oligonucleosides, also identified as MDH linked oligonucleosides, and methylenecarbonylamino linked oligonucleosides, also identified as amide-3 linked oligonucleosides, and methyleneaminocarbonyl linked oligonucleosides, also identified as amide-4 linked oligonucleosides, as well as mixed backbone compounds having, for instance, alternating MMI and P=O or P=S linkages are prepared as described in U.S. Patents 5,378,825, 5,386,023, 5,489,677, 5,602,240 and 5,610,289, all of which are herein incorporated by reference.

Formacetal and thioformacetal linked oligonucleosides are prepared as described in U.S. Patents 5,264,562 and 5,264,564, herein incorporated by reference.

Ethylene oxide linked oligonucleosides are prepared as described in U.S. Patent 5,223,618, herein incorporated by reference.

Example 3

RNA Synthesis

In general, RNA synthesis chemistry is based on the selective incorporation of various protecting groups at strategic intermediary reactions. Although one of ordinary skill in the art will understand the use of protecting groups in organic synthesis, a useful class of protecting groups includes silyl ethers. In particular bulky silyl ethers are used to protect the 5′-hydroxyl in combination with an acid-labile orthoester protecting group on the 2′-hydroxyl. This set of protecting groups is then used with standard solid-phase synthesis technology. It is important to lastly remove the acid labile orthoester protecting group after all other synthetic steps. Moreover, the early use of the silyl protecting groups during synthesis ensures facile removal when desired, without undesired deprotection of 2′ hydroxyl.

Following this procedure for the sequential protection of the 5′-hydroxyl in combination with protection of the 2′-hydroxyl by protecting groups that are differentially removed and are differentially chemically labile, RNA oligonucleotides were synthesized.

RNA oligonucleotides are synthesized in a stepwise fashion. Each nucleotide is added sequentially (3′- to 5′-direction) to a solid support-bound oligonucleotide. The first nucleoside at the 3′-end of the chain is covalently attached to a solid support. The nucleotide precursor, a ribonucleoside phosphoramidite, and activator are added, coupling the second base onto the 5′-end of the first nucleoside. The support is washed and any unreacted 5′-hydroxyl groups are capped with acetic anhydride to yield 5′-acetyl moieties. The linkage is then oxidized to the more stable and ultimately desired P(V) linkage. At the end of the nucleotide addition cycle, the 5′-silyl group is cleaved

with fluoride. The cycle is repeated for each subsequent nucleotide.

Following synthesis, the methyl protecting groups on the phosphates are cleaved in 30 minutes utilizing 1 M disodium-2-carbamoyl-2-cyanoethylene-1,1-dithiolate trihydrate (S_2Na_2) in DMF. The deprotection solution is washed from the solid support-bound oligonucleotide using water. The support is then treated with 40% methylamine in water for 10 minutes at 55 °C. This releases the RNA oligonucleotides into solution, deprotects the exocyclic amines, and modifies the 2′- groups. The oligonucleotides can be analyzed by anion exchange HPLC at this stage.

The 2′-orthoester groups are the last protecting groups to be removed. The ethylene glycol monoacetate orthoester protecting group developed by Dharmacon Research, Inc. (Lafayette, CO), is one example of a useful orthoester protecting group which, has the following important properties. It is stable to the conditions of nucleoside phosphoramidite synthesis and oligonucleotide synthesis. However, after oligonucleotide synthesis the oligonucleotide is treated with methylamine which not only cleaves the oligonucleotide from the solid support but also removes the acetyl groups from the orthoesters. The resulting 2-ethyl-hydroxyl substituents on the orthoester are less electron withdrawing than the acetylated precursor. As a result, the modified orthoester becomes more labile to acid-catalyzed hydrolysis. Specifically, the rate of cleavage is approximately 10 times faster after the acetyl groups are removed. Therefore, this orthoester possesses sufficient stability in order to be compatible with oligonucleotide synthesis and yet, when subsequently modified, permits deprotection to be carried out under relatively mild aqueous

conditions compatible with the final RNA oligonucleotide product.

Additionally, methods of RNA synthesis are well known in the art (Scaringe, S. A. Ph.D. Thesis, University of Colorado, 1996; Scaringe, S. A., et al., *J. Am. Chem. Soc.*, **1998**, *120*, 11820-11821; Matteucci, M. D. and Caruthers, M. H. *J. Am. Chem. Soc.*, **1981**, *103*, 3185-3191; Beaucage, S. L. and Caruthers, M. H. *Tetrahedron Lett.*, **1981**, *22*, 1859-1862; Dahl, B. J., et al., *Acta Chem. Scand,*. **1990**, *44*, 639-641; Reddy, M. P., et al., *Tetrahedrom Lett.*, **1994**, *25*, 4311-4314; Wincott, F. et al., *Nucleic Acids Res.*, **1995**, *23*, 2677-2684; Griffin, B. E., et al., *Tetrahedron,* **1967**, *23*, 2301-2313; Griffin, B. E., et al., *Tetrahedron,* **1967**, *23*, 2315-2331).

RNA antisense compounds (RNA oligonucleotides) of the present invention can be synthesized by the methods herein or purchased from Dharmacon Research, Inc (Lafayette, CO). Once synthesized, complementary RNA antisense compounds can then be annealed by methods known in the art to form double stranded (duplexed) antisense compounds. For example, duplexes can be formed by combining 30 µl of each of the complementary strands of RNA oligonucleotides (50 uM RNA oligonucleotide solution) and 15 µl of 5X annealing buffer (100 mM potassium acetate, 30 mM HEPES-KOH pH 7.4, 2 mM magnesium acetate) followed by heating for 1 minute at 90°C, then 1 hour at 37°C. The resulting duplexed antisense compounds can be used in kits, assays, screens, or other methods to investigate the role of a target nucleic acid.

Example 4

Synthesis of Chimeric Oligonucleotides

Chimeric oligonucleotides, oligonucleosides or mixed oligonucleotides/oligonucleosides of the invention can be of several different types. These include a first type wherein

the "gap" segment of linked nucleosides is positioned between 5' and 3' "wing" segments of linked nucleosides and a second "open end" type wherein the "gap" segment is located at either the 3' or the 5' terminus of the oligomeric compound. Oligonucleotides of the first type are also known in the art as "gapmers" or gapped oligonucleotides. Oligonucleotides of the second type are also known in the art as "hemimers" or "wingmers".

[2'-O-Me]--[2'-deoxy]--[2'-O-Me] Chimeric Phosphorothioate Oligonucleotides

Chimeric oligonucleotides having 2'-O-alkyl phosphorothioate and 2'-deoxy phosphorothioate oligonucleotide segments are synthesized using an Applied Biosystems automated DNA synthesizer Model 394, as above. Oligonucleotides are synthesized using the automated synthesizer and 2'-deoxy-5'-dimethoxytrityl-3'-O-phosphoramidite for the DNA portion and 5'-dimethoxytrityl-2'-O-methyl-3'-O-phosphoramidite for 5' and 3' wings. The standard synthesis cycle is modified by incorporating coupling steps with increased reaction times for the 5'-dimethoxytrityl-2'-O-methyl-3'-O-phosphoramidite. The fully protected oligonucleotide is cleaved from the support and deprotected in concentrated ammonia (NH_4OH) for 12-16 hr at 55ºC. The deprotected oligo is then recovered by an appropriate method (precipitation, column chromatography, volume reduced *in vacuo* and analyzed spetrophotometrically for yield and for purity by capillary electrophoresis and by mass spectrometry.

[2'-O-(2-Methoxyethyl)]--[2'-deoxy]--[2'-O-(Methoxyethyl)] Chimeric Phosphorothioate Oligonucleotides

[2'-O-(2-methoxyethyl)]--[2'-deoxy]--[-2'-O-

(methoxyethyl)] chimeric phosphorothioate oligonucleotides were prepared as per the procedure above for the 2'-O-methyl chimeric oligonucleotide, with the substitution of 2'-O-(methoxyethyl) amidites for the 2'-O-methyl amidites.

[2'-O-(2-Methoxyethyl)Phosphodiester]--[2'-deoxy Phosphorothioate]--[2'-O-(2-Methoxyethyl) Phosphodiester] Chimeric Oligonucleotides

[2'-O-(2-methoxyethyl phosphodiester]--[2'-deoxy phosphorothioate]--[2'-O-(methoxyethyl) phosphodiester] chimeric oligonucleotides are prepared as per the above procedure for the 2'-O-methyl chimeric oligonucleotide with the substitution of 2'-O-(methoxyethyl) amidites for the 2'-O-methyl amidites, oxidation with iodine to generate the phosphodiester internucleotide linkages within the wing portions of the chimeric structures and sulfurization utilizing 3,H-1,2 benzodithiole-3-one 1,1 dioxide (Beaucage Reagent) to generate the phosphorothioate internucleotide linkages for the center gap.

Other chimeric oligonucleotides, chimeric oligonucleosides and mixed chimeric oligonucleotides/oligonucleosides are synthesized according to United States patent 5,623,065, herein incorporated by reference.

Example 5

Design and screening of duplexed antisense compounds targeting diacylglycerol acyltransferase 2

In accordance with the present invention, a series of nucleic acid duplexes comprising the antisense compounds of the present invention and their complements can be designed to target diacylglycerol acyltransferase 2. The nucleobase sequence of the antisense strand of the duplex comprises at

least an 8-nucleobase portion of an oligonucleotide in Table 1. The ends of the strands may be modified by the addition of one or more natural or modified nucleobases to form an overhang. The sense strand of the dsRNA is then designed and synthesized as the complement of the antisense strand and may also contain modifications or additions to either terminus. For example, in one embodiment, both strands of the dsRNA duplex would be complementary over the central nucleobases, each having overhangs at one or both termini.

For example, a duplex comprising an antisense strand having the sequence CGAGAGGCGGACGGGACCG and having a two-nucleobase overhang of deoxythymidine(dT) would have the following structure:

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    cgagaggcggacgggaccgTT    Antisense Strand
    ||||||||||||||||||||
TTgctctccgcctgccctggc      Complement
```

RNA strands of the duplex can be synthesized by methods disclosed herein or purchased from Dharmacon Research Inc., (Lafayette, CO). Once synthesized, the complementary strands are annealed. The single strands are aliquoted and diluted to a concentration of 50 uM. Once diluted, 30 uL of each strand is combined with 15uL of a 5X solution of annealing buffer. The final concentration of said buffer is 100 mM potassium acetate, 30 mM HEPES-KOH pH 7.4, and 2mM magnesium acetate. The final volume is 75 uL. This solution is incubated for 1 minute at 90°C and then centrifuged for 15 seconds. The tube is allowed to sit for 1 hour at 37°C at which time the dsRNA duplexes are used in experimentation. The final concentration of the dsRNA duplex is 20 uM. This solution can be stored frozen (-20°C) and freeze-thawed up to 5 times.

Once prepared, the duplexed antisense compounds are evaluated for their ability to modulate diacylglycerol acyltransferase 2 expression.

When cells reached 80% confluency, they are treated with duplexed antisense compounds of the invention. For cells grown in 96-well plates, wells are washed once with 200 µL OPTI-MEM-1 reduced-serum medium (Gibco BRL) and then treated with 130 µL of OPTI-MEM-1 containing 12 µg/mL LIPOFECTIN (Gibco BRL) and the desired duplex antisense compound at a final concentration of 200 nM. After 5 hours of treatment, the medium is replaced with fresh medium. Cells are harvested 16 hours after treatment, at which time RNA is isolated and target reduction measured by RT-PCR.

Example 6

Oligonucleotide Isolation

After cleavage from the controlled pore glass solid support and deblocking in concentrated ammonium hydroxide at 55?C for 12-16 hours, the oligonucleotides or oligonucleosides are recovered by precipitation out of 1 M NH_4OAc with >3 volumes of ethanol. Synthesized oligonucleotides were analyzed by electrospray mass spectroscopy (molecular weight determination) and by capillary gel electrophoresis and judged to be at least 70% full length material. The relative amounts of phosphorothioate and phosphodiester linkages obtained in the synthesis was determined by the ratio of correct molecular weight relative to the -16 amu product (+/- 32 +/-48). For some studies oligonucleotides were purified by HPLC, as described by Chiang *et al.*, *J. Biol. Chem.* **1991**, *266*, 18162-18171. Results obtained with HPLC-purified material were similar to those obtained with non-HPLC purified material.

Example 7

Oligonucl otide Synthesis - 96 Well Plate Format

Oligonucleotides were synthesized via solid phase P(III)

phosphoramidite chemistry on an automated synthesizer capable of assembling 96 sequences simultaneously in a 96-well format. Phosphodiester internucleotide linkages were afforded by oxidation with aqueous iodine. Phosphorothioate internucleotide linkages were generated by sulfurization utilizing 3,H-1,2 benzodithiole-3-one 1,1 dioxide (Beaucage Reagent) in anhydrous acetonitrile. Standard base-protected beta-cyanoethyl-diiso-propyl phosphoramidites were purchased from commercial vendors (e.g. PE-Applied Biosystems, Foster City, CA, or Pharmacia, Piscataway, NJ). Non-standard nucleosides are synthesized as per standard or patented methods. They are utilized as base protected beta-cyanoethyldiisopropyl phosphoramidites.

Oligonucleotides were cleaved from support and deprotected with concentrated NH_4OH at elevated temperature (55-60°C) for 12-16 hours and the released product then dried *in vacuo*. The dried product was then re-suspended in sterile water to afford a master plate from which all analytical and test plate samples are then diluted utilizing robotic pipettors.

Example 8

Oligonucleotide Analysis - 96-Well Plate Format

The concentration of oligonucleotide in each well was assessed by dilution of samples and UV absorption spectroscopy. The full-length integrity of the individual products was evaluated by capillary electrophoresis (CE) in either the 96-well format (Beckman P/ACE? MDQ) or, for individually prepared samples, on a commercial CE apparatus (e.g., Beckman P/ACE? 5000, ABI 270). Base and backbone composition was confirmed by mass analysis of the compounds utilizing electrospray-mass spectroscopy. All assay test plates were diluted from the master plate using single and

multi-channel robotic pipettors. Plates were judged to be acceptable if at least 85% of the compounds on the plate were at least 85% full length.

Example 9

Cell culture and oligonucleotide treatment

The effect of antisense compounds on target nucleic acid expression can be tested in any of a variety of cell types provided that the target nucleic acid is present at measurable levels. This can be routinely determined using, for example, PCR or Northern blot analysis. The following cell types are provided for illustrative purposes, but other cell types can be routinely used, provided that the target is expressed in the cell type chosen. This can be readily determined by methods routine in the art, for example Northern blot analysis, ribonuclease protection assays, or RT-PCR.

T-24 cells:

The human transitional cell bladder carcinoma cell line T-24 was obtained from the American Type Culture Collection (ATCC) (Manassas, VA). T-24 cells were routinely cultured in complete McCoy's 5A basal media (Invitrogen Corporation, Carlsbad, CA) supplemented with 10% fetal calf serum (Invitrogen Corporation, Carlsbad, CA), penicillin 100 units per mL, and streptomycin 100 micrograms per mL (Invitrogen Corporation, Carlsbad, CA). Cells were routinely passaged by trypsinization and dilution when they reached 90% confluence. Cells were seeded into 96-well plates (Falcon-Primaria #353872) at a density of 7000 cells/well for use in RT-PCR analysis.

For Northern blotting or other analysis, cells may be seeded onto 100 mm or other standard tissue culture plates

and treated similarly, using appropriate volumes of medium and oligonucleotide.

A549 cells:

The human lung carcinoma cell line A549 was obtained from the American Type Culture Collection (ATCC) (Manassas, VA). A549 cells were routinely cultured in DMEM basal media (Invitrogen Corporation, Carlsbad, CA) supplemented with 10% fetal calf serum (Invitrogen Corporation, Carlsbad, CA), penicillin 100 units per mL, and streptomycin 100 micrograms per mL (Invitrogen Corporation, Carlsbad, CA). Cells were routinely passaged by trypsinization and dilution when they reached 90% confluence.

NHDF cells:

Human neonatal dermal fibroblast (NHDF) were obtained from the Clonetics Corporation (Walkersville, MD). NHDFs were routinely maintained in Fibroblast Growth Medium (Clonetics Corporation, Walkersville, MD) supplemented as recommended by the supplier. Cells were maintained for up to 10 passages as recommended by the supplier.

HEK cells:

Human embryonic keratinocytes (HEK) were obtained from the Clonetics Corporation (Walkersville, MD). HEKs were routinely maintained in Keratinocyte Growth Medium (Clonetics Corporation, Walkersville, MD) formulated as recommended by the supplier. Cells were routinely maintained for up to 10 passages as recommended by the supplier.

3T3-L1 cells:

The mouse embryonic adipocyte-like cell line 3T3-L1 was

obtained from the American Type Culture Collection (Manassas, VA). 3T3-L1 cells were routinely cultured in DMEM, high glucose (Gibco/Life Technologies, Gaithersburg, MD) supplemented with 10% fetal calf serum (Gibco/Life Technologies, Gaithersburg, MD). Cells were routinely passaged by trypsinization and dilution when they reached 80% confluence. Cells were seeded into 96-well plates (Falcon-Primaria #3872) at a density of 4000 cells/well for use in RT-PCR analysis.

For Northern blotting or other analyses, cells may be seeded onto 100 mm or other standard tissue culture plates and treated similarly, using appropriate volumes of medium and oligonucleotide.

Treatment with antisense compounds:

When cells reached 65-75% confluency, they were treated with oligonucleotide. For cells grown in 96-well plates, wells were washed once with 100 ?L OPTI-MEM? -1 reduced-serum medium (Invitrogen Corporation, Carlsbad, CA) and then treated with 130 ?L of OPTI-MEM? -1 containing 3.75 ?g/mL LIPOFECTIN? (Invitrogen Corporation, Carlsbad, CA) and the desired concentration of oligonucleotide. Cells are treated and data are obtained in triplicate. After 4-7 hours of treatment at 37°C, the medium was replaced with fresh medium. Cells were harvested 16-24 hours after oligonucleotide treatment.

The concentration of oligonucleotide used varies from cell line to cell line. To determine the optimal oligonucleotide concentration for a particular cell line, the cells are treated with a positive control oligonucleotide at a range of concentrations. For human cells the positive control oligonucleotide is selected from either ISIS 13920 (**TCC**GTCATCGCT**CCTCAGGG**, SEQ ID NO: 1) which is targeted to

human H-ras, or ISIS 18078, (**GTGCG**CGCGAGCCCG**AAATC**, SEQ ID NO: 2) which is targeted to human Jun-N-terminal kinase-2 (JNK2). Both controls are 2'-O-methoxyethyl gapmers (2'-O-methoxyethyls shown in bold) with a phosphorothioate backbone. For mouse or rat cells the positive control oligonucleotide is ISIS 15770, **ATGCA**TTCTGCCCCC**AAGGA**, SEQ ID NO: 3, a 2'-O-methoxyethyl gapmer (2'-O-methoxyethyls shown in bold) with a phosphorothioate backbone which is targeted to both mouse and rat c-raf. The concentration of positive control oligonucleotide that results in 80% inhibition of c-H-ras (for ISIS 13920), JNK2 (for ISIS 18078) or c-raf (for ISIS 15770) mRNA is then utilized as the screening concentration for new oligonucleotides in subsequent experiments for that cell line. If 80% inhibition is not achieved, the lowest concentration of positive control oligonucleotide that results in 60% inhibition of c-H-ras, JNK2 or c-raf mRNA is then utilized as the oligonucleotide screening concentration in subsequent experiments for that cell line. If 60% inhibition is not achieved, that particular cell line is deemed as unsuitable for oligonucleotide transfection experiments. The concentrations of antisense oligonucleotides used herein are from 50 nM to 300 nM.

Example 10

Analysis of oligonucleotide inhibition of diacylglycerol acyltransferase 2 expression

Antisense modulation of diacylglycerol acyltransferase 2 expression can be assayed in a variety of ways known in the art. For example, diacylglycerol acyltransferase 2 mRNA levels can be quantitated by, e.g., Northern blot analysis, competitive polymerase chain reaction (PCR), or real-time PCR (RT-PCR). Real-time quantitative PCR is presently preferred.

RNA analysis can be performed on total cellular RNA or poly(A)+ mRNA. The preferred method of RNA analysis of the present invention is the use of total cellular RNA as described in other examples herein. Methods of RNA isolation are well known in the art. Northern blot analysis is also routine in the art. Real-time quantitative (PCR) can be conveniently accomplished using the commercially available ABI PRISM? 7600, 7700, or 7900 Sequence Detection System, available from PE-Applied Biosystems, Foster City, CA and used according to manufacturer's instructions.

Protein levels of diacylglycerol acyltransferase 2 can be quantitated in a variety of ways well known in the art, such as immunoprecipitation, Western blot analysis (immunoblotting), enzyme-linked immunosorbent assay (ELISA) or fluorescence-activated cell sorting (FACS). Antibodies directed to diacylglycerol acyltransferase 2 can be identified and obtained from a variety of sources, such as the MSRS catalog of antibodies (Aerie Corporation, Birmingham, MI), or can be prepared via conventional monoclonal or polyclonal antibody generation methods well known in the art.

Example 11

Design of phenotypic assays and *in vivo* studies for the use of diacylglycerol acyltransferase 2 inhibitors

Phenotypic assays

Once diacylglycerol acyltransferase 2 inhibitors have been identified by the methods disclosed herein, the compounds are further investigated in one or more phenotypic assays, each having measurable endpoints predictive of efficacy in the treatment of a particular disease state or condition.

Phenotypic assays, kits and reagents for their use are well known to those skilled in the art and are herein used to investigate the role and/or association of diacylglycerol acyltransferase 2 in health and disease. Representative phenotypic assays, which can be purchased from any one of several commercial vendors, include those for determining cell viability, cytotoxicity, proliferation or cell survival (Molecular Probes, Eugene, OR; PerkinElmer, Boston, MA), protein-based assays including enzymatic assays (Panvera, LLC, Madison, WI; BD Biosciences, Franklin Lakes, NJ; Oncogene Research Products, San Diego, CA), cell regulation, signal transduction, inflammation, oxidative processes and apoptosis (Assay Designs Inc., Ann Arbor, MI), triglyceride accumulation (Sigma-Aldrich, St. Louis, MO), angiogenesis assays, tube formation assays, cytokine and hormone assays and metabolic assays (Chemicon International Inc., Temecula, CA; Amersham Biosciences, Piscataway, NJ).

In one non-limiting example, cells determined to be appropriate for a particular phenotypic assay (i.e., MCF-7 cells selected for breast cancer studies; adipocytes for obesity studies) are treated with diacylglycerol acyltransferase 2 inhibitors identified from the *in vitro* studies as well as control compounds at optimal concentrations which are determined by the methods described above. At the end of the treatment period, treated and untreated cells are analyzed by one or more methods specific for the assay to determine phenotypic outcomes and endpoints.

Phenotypic endpoints include changes in cell morphology over time or treatment dose as well as changes in levels of cellular components such as proteins, lipids, nucleic acids, hormones, saccharides or metals. Measurements of cellular status which include pH, stage of the cell cycle, intake or

excretion of biological indicators by the cell, are also endpoints of interest.

Analysis of the geneotype of the cell (measurement of the expression of one or more of the genes of the cell) after treatment is also used as an indicator of the efficacy or potency of the diacylglycerol acyltransferase 2 inhibitors. Hallmark genes, or those genes suspected to be associated with a specific disease state, condition, or phenotype, are measured in both treated and untreated cells.

In vivo studies

The individual subjects of the *in vivo* studies described herein are warm-blooded vertebrate animals, which includes humans.

The clinical trial is subjected to rigorous controls to ensure that individuals are not unnecessarily put at risk and that they are fully informed about their role in the study. To account for the psychological effects of receiving treatments, volunteers are randomly given placebo or diacylglycerol acyltransferase 2 inhibitor. Furthermore, to prevent the doctors from being biased in treatments, they are not informed as to whether the medication they are administering is a diacylglycerol acyltransferase 2 inhibitor or a placebo. Using this randomization approach, each volunteer has the same chance of being given either the new treatment or the placebo.

Volunteers receive either the diacylglycerol acyltransferase 2 inhibitor or placebo for eight week period with biological parameters associated with the indicated disease state or condition being measured at the beginning (baseline measurements before any treatment), end (after the final treatment), and at regular intervals during the study period. Such measurements include the levels of nucleic acid

molecules encoding diacylglycerol acyltransferase 2 or diacylglycerol acyltransferase 2 protein levels in body fluids, tissues or organs compared to pre-treatment levels. Other measurements include, but are not limited to, indices of the disease state or condition being treated, body weight, blood pressure, serum titers of pharmacologic indicators of disease or toxicity as well as ADME (absorption, distribution, metabolism and excretion) measurements.

Information recorded for each patient includes age (years), gender, height (cm), family history of disease state or condition (yes/no), motivation rating (some/moderate/great) and number and type of previous treatment regimens for the indicated disease or condition.

Volunteers taking part in this study are healthy adults (age 18 to 65 years) and roughly an equal number of males and females participate in the study. Volunteers with certain characteristics are equally distributed for placebo and diacylglycerol acyltransferase 2 inhibitor treatment. In general, the volunteers treated with placebo have little or no response to treatment, whereas the volunteers treated with the diacylglycerol acyltransferase 2 inhibitor show positive trends in their disease state or condition index at the conclusion of the study.

Example 12

RNA Isolation

Poly(A)+ mRNA isolation

Poly(A)+ mRNA was isolated according to Miura *et al.*, (*Clin. Chem.*, **1996**, *42*, 1758-1764). Other methods for poly(A)+ mRNA isolation are routine in the art. Briefly, for cells grown on 96-well plates, growth medium was removed from the cells and each well was washed with 200 ?L cold PBS. 60 ?L lysis buffer (10 mM Tris-HCl, pH 7.6, 1 mM EDTA, 0.5 M

NaCl, 0.5% NP-40, 20 mM vanadyl-ribonucleoside complex) was added to each well, the plate was gently agitated and then incubated at room temperature for five minutes. 55 ?L of lysate was transferred to Oligo d(T) coated 96-well plates (AGCT Inc., Irvine CA). Plates were incubated for 60 minutes at room temperature, washed 3 times with 200 ?L of wash buffer (10 mM Tris-HCl pH 7.6, 1 mM EDTA, 0.3 M NaCl). After the final wash, the plate was blotted on paper towels to remove excess wash buffer and then air-dried for 5 minutes. 60 ?L of elution buffer (5 mM Tris-HCl pH 7.6), preheated to 70?C, was added to each well, the plate was incubated on a 90?C hot plate for 5 minutes, and the eluate was then transferred to a fresh 96-well plate.

Cells grown on 100 mm or other standard plates may be treated similarly, using appropriate volumes of all solutions.

Total RNA Isolation

Total RNA was isolated using an RNEASY 96? kit and buffers purchased from Qiagen Inc. (Valencia, CA) following the manufacturer's recommended procedures. Briefly, for cells grown on 96-well plates, growth medium was removed from the cells and each well was washed with 200 ?L cold PBS. 150 ?L Buffer RLT was added to each well and the plate vigorously agitated for 20 seconds. 150 ?L of 70% ethanol was then added to each well and the contents mixed by pipetting three times up and down. The samples were then transferred to the RNEASY 96? well plate attached to a QIAVAC? manifold fitted with a waste collection tray and attached to a vacuum source. Vacuum was applied for 1 minute. 500 ?L of Buffer RW1 was added to each well of the RNEASY 96? plate and incubated for 15 minutes and the vacuum was again applied for 1 minute. An

additional 500 ?L of Buffer RW1 was added to each well of the RNEASY 96? plate and the vacuum was applied for 2 minutes. 1 mL of Buffer RPE was then added to each well of the RNEASY 96? plate and the vacuum applied for a period of 90 seconds. The Buffer RPE wash was then repeated and the vacuum was applied for an additional 3 minutes. The plate was then removed from the QIAVAC? manifold and blotted dry on paper towels. The plate was then re-attached to the QIAVAC? manifold fitted with a collection tube rack containing 1.2 mL collection tubes. RNA was then eluted by pipetting 140 ?L of RNAse free water into each well, incubating 1 minute, and then applying the vacuum for 3 minutes.

The repetitive pipetting and elution steps may be automated using a QIAGEN Bio-Robot 9604 (Qiagen, Inc., Valencia CA). Essentially, after lysing of the cells on the culture plate, the plate is transferred to the robot deck where the pipetting, DNase treatment and elution steps are carried out.

Example 13

Real-time Quantitative PCR Analysis of diacylglycerol acyltransferase 2 mRNA Levels

Quantitation of diacylglycerol acyltransferase 2 mRNA levels was accomplished by real-time quantitative PCR using the ABI PRISM? 7600, 7700, or 7900 Sequence Detection System (PE-Applied Biosystems, Foster City, CA) according to manufacturer's instructions. This is a closed-tube, non-gel-based, fluorescence detection system which allows high-throughput quantitation of polymerase chain reaction (PCR) products in real-time. As opposed to standard PCR in which amplification products are quantitated after the PCR is completed, products in real-time quantitative PCR are

quantitated as they accumulate. This is accomplished by including in the PCR reaction an oligonucleotide probe that anneals specifically between the forward and reverse PCR primers, and contains two fluorescent dyes. A reporter dye (e.g., FAM or JOE, obtained from either PE-Applied Biosystems, Foster City, CA, Operon Technologies Inc., Alameda, CA or Integrated DNA Technologies Inc., Coralville, IA) is attached to the 5' end of the probe and a quencher dye (e.g., TAMRA, obtained from either PE-Applied Biosystems, Foster City, CA, Operon Technologies Inc., Alameda, CA or Integrated DNA Technologies Inc., Coralville, IA) is attached to the 3' end of the probe. When the probe and dyes are intact, reporter dye emission is quenched by the proximity of the 3' quencher dye. During amplification, annealing of the probe to the target sequence creates a substrate that can be cleaved by the 5'-exonuclease activity of Taq polymerase. During the extension phase of the PCR amplification cycle, cleavage of the probe by Taq polymerase releases the reporter dye from the remainder of the probe (and hence from the quencher moiety) and a sequence-specific fluorescent signal is generated. With each cycle, additional reporter dye molecules are cleaved from their respective probes, and the fluorescence intensity is monitored at regular intervals by laser optics built into the ABI PRISM? Sequence Detection System. In each assay, a series of parallel reactions containing serial dilutions of mRNA from untreated control samples generates a standard curve that is used to quantitate the percent inhibition after antisense oligonucleotide treatment of test samples.

Prior to quantitative PCR analysis, primer-probe sets specific to the target gene being measured are evaluated for their ability to be "multiplexed" with a GAPDH amplification reaction. In multiplexing, both the target gene and the

internal standard gene GAPDH are amplified concurrently in a single sample. In this analysis, mRNA isolated from untreated cells is serially diluted. Each dilution is amplified in the presence of primer-probe sets specific for GAPDH only, target gene only ("single-plexing"), or both (multiplexing). Following PCR amplification, standard curves of GAPDH and target mRNA signal as a function of dilution are generated from both the single-plexed and multiplexed samples. If both the slope and correlation coefficient of the GAPDH and target signals generated from the multiplexed samples fall within 10% of their corresponding values generated from the single-plexed samples, the primer-probe set specific for that target is deemed multiplexable. Other methods of PCR are also known in the art.

PCR reagents were obtained from Invitrogen Corporation, (Carlsbad, CA). RT-PCR reactions were carried out by adding 20 ?L PCR cocktail (2.5x PCR buffer minus $MgCl_2$, 6.6 mM $MgCl_2$, 375 ?M each of dATP, dCTP, dCTP and dGTP, 375 nM each of forward primer and reverse primer, 125 nM of probe, 4 Units RNAse inhibitor, 1.25 Units PLATINUM® Taq, 5 Units MuLV reverse transcriptase, and 2.5x ROX dye) to 96-well plates containing 30 ?L total RNA solution (20-200 ng). The RT reaction was carried out by incubation for 30 minutes at 48?C. Following a 10 minute incubation at 95?C to activate the PLATINUM® Taq, 40 cycles of a two-step PCR protocol were carried out: 95?C for 15 seconds (denaturation) followed by 60?C for 1.5 minutes (annealing/extension).

Gene target quantities obtained by real time RT-PCR are normalized using either the expression level of GAPDH, a gene whose expression is constant, or by quantifying total RNA using RiboGreen™ (Molecular Probes, Inc. Eugene, OR). GAPDH expression is quantified by real time RT-PCR, by being run

simultaneously with the target, multiplexing, or separately. Total RNA is quantified using RiboGreen™ RNA quantification reagent (Molecular Probes, Inc. Eugene, OR). Methods of RNA quantification by RiboGreen™ are taught in Jones, L.J., et al, (Analytical Biochemistry, 1998, 265, 368-374).

In this assay, 170 ?L of RiboGreen™ working reagent (RiboGreen™ reagent diluted 1:350 in 10mM Tris-HCl, 1 mM EDTA, pH 7.5) is pipetted into a 96-well plate containing 30 ?L purified, cellular RNA. The plate is read in a CytoFluor 4000 (PE Applied Biosystems) with excitation at 485nm and emission at 530nm.

Probes and primers to human diacylglycerol acyltransferase 2 were designed to hybridize to a human diacylglycerol acyltransferase 2 sequence, using published sequence information (GenBank accession number NM_032564.2, incorporated herein as SEQ ID NO: 4). For human diacylglycerol acyltransferase 2 the PCR primers were: forward primer: CATACGGCCTTACCTGGCTACA (SEQ ID NO: 5) reverse primer: CAGACATCAGGTACTCCCTCAACA (SEQ ID NO: 6) and the PCR probe was: FAM-TGGCAGGCAACTTCCGAATGCC-TAMRA (SEQ ID NO: 7) where FAM is the fluorescent dye and TAMRA is the quencher dye. For human GAPDH the PCR primers were: forward primer: GAAGGTGAAGGTCGGAGTC(SEQ ID NO:8) reverse primer: GAAGATGGTGATGGGATTTC (SEQ ID NO:9) and the PCR probe was: 5' JOE-CAAGCTTCCCGTTCTCAGCC- TAMRA 3' (SEQ ID NO: 10) where JOE is the fluorescent reporter dye and TAMRA is the quencher dye.

Probes and primers to mouse diacylglycerol acyltransferase 2 were designed to hybridize to a mouse diacylglycerol acyltransferase 2 sequence, using published sequence information (GenBank accession number AK002443.1, incorporated herein as SEQ ID NO:11). For mouse diacylglycerol acyltransferase 2 the PCR primers were:

forward primer: ACTCTGGAGGTTGGCACCAT (SEQ ID NO:12)
reverse primer: GGGTGTGGCTCAGGAGGAT (SEQ ID NO: 13) and the PCR probe was: FAM-CAGCGTTGCTCTGGCGCA-TAMRA (SEQ ID NO: 14) where FAM is the fluorescent reporter dye and TAMRA is the quencher dye. For mouse GAPDH the PCR primers were:
forward primer: GGCAAATTCAACGGCACAGT(SEQ ID NO:15)
reverse primer: GGGTCTCGCTCCTGGAAGAT(SEQ ID NO:16) and the PCR probe was: 5' JOE-AAGGCCGAGAATGGGAAGCTTGTCATC- TAMRA 3' (SEQ ID NO: 17) where JOE is the fluorescent reporter dye and TAMRA is the quencher dye.

Example 14

Northern blot analysis of diacylglycerol acyltransferase 2 mRNA levels

Eighteen hours after antisense treatment, cell monolayers were washed twice with cold PBS and lysed in 1 mL RNAZOL? (TEL-TEST "B" Inc., Friendswood, TX). Total RNA was prepared following manufacturer's recommended protocols. Twenty micrograms of total RNA was fractionated by electrophoresis through 1.2% agarose gels containing 1.1% formaldehyde using a MOPS buffer system (AMRESCO, Inc. Solon, OH). RNA was transferred from the gel to HYBOND? -N+ nylon membranes (Amersham Pharmacia Biotech, Piscataway, NJ) by overnight capillary transfer using a Northern/Southern Transfer buffer system (TEL-TEST "B" Inc., Friendswood, TX). RNA transfer was confirmed by UV visualization. Membranes were fixed by UV cross-linking using a STRATALINKER? UV Crosslinker 2400 (Stratagene, Inc, La Jolla, CA) and then probed using QUICKHYB? hybridization solution (Stratagene, La Jolla, CA) using manufacturer's recommendations for stringent conditions.

To detect human diacylglycerol acyltransferase 2, a human diacylglycerol acyltransferase 2 specific probe was prepared by PCR using the forward primer CATACGGCCTTACCTGGCTACA (SEQ ID NO: 5) and the reverse primer CAGACATCAGGTACTCCCTCAACA (SEQ ID NO: 6). To normalize for variations in loading and transfer efficiency membranes were stripped and probed for human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) RNA (Clontech, Palo Alto, CA).

To detect mouse diacylglycerol acyltransferase 2, a mouse diacylglycerol acyltransferase 2 specific probe was prepared by PCR using the forward primer ACTCTGGAGGTTGGCACCAT (SEQ ID NO: 12) and the reverse primer GGGTGTGGCTCAGGAGGAT (SEQ ID NO: 13). To normalize for variations in loading and transfer efficiency membranes were stripped and probed for mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) RNA (Clontech, Palo Alto, CA).

Hybridized membranes were visualized and quantitated using a PHOSPHORIMAGER? and IMAGEQUANT? Software V3.3 (Molecular Dynamics, Sunnyvale, CA). Data was normalized to GAPDH levels in untreated controls.

Example 15

Antisense inhibition of human diacylglycerol acyltransferase 2 expression by chimeric phosphorothioate oligonucleotides having 2'-MOE wings and a deoxy gap

In accordance with the present invention, a series of antisense compounds was designed to target different regions of the human diacylglycerol acyltransferase 2 RNA, using published sequences (GenBank accession number NM_032564.2, incorporated herein as SEQ ID NO: 4, nucleotides 5669186 to 5712008 of the nucleotide sequence with the GenBank accession number NT_033927.5, incorporated herein as SEQ ID NO: 18).

The compounds are shown in Table 1. "Target site" indicates the first (5'-most) nucleotide number on the particular target sequence to which the compound binds. All compounds in Table 1 are chimeric oligonucleotides ("gapmers") 20 nucleotides in length, composed of a central "gap" region consisting of ten 2'-deoxynucleotides, which is flanked on both sides (5' and 3' directions) by five-nucleotide "wings". The wings are composed of 2'-methoxyethyl (2'-MOE)nucleotides. The internucleoside (backbone) linkages are phosphorothioate (P=S) throughout the oligonucleotide. All cytidine residues are 5-methylcytidines. The compounds were analyzed for their effect on human diacylglycerol acyltransferase 2 mRNA levels by quantitative real-time PCR as described in other examples herein. Data are averages from three experiments in which A549 cells were treated with the antisense oligonucleotides of the present invention. The positive control for each datapoint is identified in the table by sequence ID number. If present, "N.D." indicates "no data".

Table 1

Inhibition of human diacylglycerol acyltransferase 2 mRNA levels by chimeric phosphorothioate oligonucleotides having 2'-MOE wings and a deoxy gap

ISIS #	REGION	TARGET SEQ ID NO	TARGET SITE	SEQUENCE	% INHIB	SEQ ID NO	CONTROL SEQ ID NO
217310	Coding	4	579	ctcctgccacctttcttggg	79	20	1
217312	Coding	4	639	tggatgggaaagtagtctcg	82	21	1
217313	Coding	4	644	ccagctggatgggaaagtag	34	22	1
217314	Coding	4	649	cttcaccagctggatgggaa	40	23	1
217315	Coding	4	654	tgtgtcttcaccagctggat	86	24	1
217316	Coding	4	659	ggttgtgtgtcttcaccagc	88	25	1
217317	Coding	4	664	cagcaggttgtgtgtcttca	93	26	1
217318	Coding	4	669	gtggtcagcaggttgtgtgt	74	27	1
217319	Coding	4	674	tcctggtggtcagcaggttg	84	28	1
217320	Coding	4	679	atagttcctggtggtcagca	90	29	1

217321	Coding	4	684	aagatatagttcctggtggt	77	30	1
217322	Coding	4	689	atccaaagatatagttcctg	73	31	1
217323	Coding	4	694	gtggtatccaaagatatagt	70	32	1
217324	Coding	4	723	aaggcacccaggcccatgat	74	33	1
217325	Coding	4	846	cctccagacatcaggtactc	73	34	1
217328	Coding	4	909	gcattgccactcccattctt	89	35	1
217329	Coding	4	914	tgatagcattgccactccca	88	36	1
217330	Coding	4	919	gatgatgatagcattgccac	77	37	1
217331	Coding	4	924	accacgatgatgatagcatt	77	38	1
217333	Coding	4	963	ttgccaggcatggagctcag	79	39	1
217336	Coding	4	1110	tggacccatcggccccagga	72	40	1
217337	Coding	4	1115	tcttctggacccatcggccc	76	41	1
217338	Coding	4	1120	gaacttcttctggacccatc	43	42	1
217339	Coding	4	1125	ttctggaacttcttctggac	62	43	1
217341	Coding	4	1197	ggcaccagcccccaggtgtc	68	44	1
217342	Coding	4	1202	agtagggcaccagcccccag	54	45	1
217343	Coding	4	1207	cttggagtagggcaccagcc	69	46	1
217346	Coding	4	1309	cagggcctccatgtacatgg	81	47	1
217347	Coding	4	1314	ttcaccagggcctccatgta	54	48	1
217348	Coding	4	1319	agagcttcaccagggcctcc	83	49	1
217353	3'UTR	4	1469	aacccacagacacccatgac	65	50	1
217354	3'UTR	4	1474	taaataacccacagacaccc	40	51	1
217355	3'UTR	4	1479	tcttttaaataacccacaga	47	52	1
334165	intron	18	21985	acaaaagagcatcctcctca	64	53	1
334166	intron	18	23110	actataaatgcttcagtcca	78	54	1
334167	exon:intron	18	31175	ttgcacttacctttcttggg	8	55	1
334168	exon:intron	18	31611	agcactttacctggatggga	63	56	1
334169	intron	18	33686	tcagtgaaatgaggcagatg	84	57	1
334170	intron	18	35303	ctcaaaagaggtgacatcaa	72	58	1
334171	exon:intron	18	37412	ggattcttacctccagacat	22	59	1
334172	intron:exon	18	39106	caggtcagctctggaaggga	47	60	1
334173	intron	18	37108	ttcccctggacctccatggg	76	61	1
334174	5'UTR	4	46	gtggcgcgagagaaacagcc	82	62	1
334175	5'UTR	4	134	gccagggcttcgcgcagagc	75	63	1
334176	Start Codon	4	222	agggtcttcatggctgaagc	53	64	1
334177	Coding	4	246	aggaccccggagtaggcggc	95	65	1
334178	Coding	4	441	acccactggagcactgagat	83	66	1
334179	Coding	4	855	gggcagatacctccagacat	28	67	1
334180	Coding	4	987	cggttccgcagggtgactgc	72	68	1
334181	Stop Codon	4	1387	aaggctggctcagttcacct	78	69	1
334182	3'UTR	4	1401	gggagttggccccgaaggct	64	70	1
334183	3'UTR	4	1414	gctggttcctccagggagtt	81	71	1
334184	3'UTR	4	1449	acttccaaatttacagagca	72	72	1
334185	3'UTR	4	1584	ccacctagaacagggcaagc	80	73	1
334186	3'UTR	4	1635	gggaagaagagaggttagct	35	74	1
334187	3'UTR	4	1647	tcacttcaggaagggaagaa	63	75	1
334188	3'UTR	4	1679	ccttcttccccaagaagact	51	76	1
334189	3'UTR	4	1707	ctaactggtccaagtcacta	82	77	1
334190	3'UTR	4	1724	ggcaaaaagtgaatcatcta	76	78	1
334191	3'UTR	4	1743	ttcgcctctcatccctaggg	13	79	1
334192	3'UTR	4	1763	ggcttgtatgagaagtggct	77	80	1
334193	3'UTR	4	1802	tttcaggactagacgagcgt	82	81	1
334194	3'UTR	4	1946	ctccgatatgagtgactagg	85	82	1
334195	3'UTR	4	1969	ctcatcctggaggccagtcc	72	83	1
334196	3'UTR	4	1974	ccatcctcatcctggaggcc	50	84	1
334197	3'UTR	4	1989	gtgtcattgccacccccatc	49	85	1

334198	3'UTR	4	2055	acctagctcatggtggcggc	67	86	1
334199	3'UTR	4	2067	accagttactccacctagct	73	87	1
334200	3'UTR	4	2088	gtcatcagccacccaagaaa	73	88	1
334201	3'UTR	4	2125	gtgctccaggccaaggctga	75	89	1
334202	3'UTR	4	2137	accagtaagcatgtgctcca	84	90	1
334203	3'UTR	4	2143	gaggccaccagtaagcatgt	65	91	1
334204	3'UTR	4	2150	gtaaactgaggccaccagta	82	92	1
334205	3'UTR	4	2184	cttcctcacatccagaatct	22	93	1
334206	3'UTR	4	2220	tgctcagaaggccaggcccc	89	94	1
334207	3'UTR	4	2242	acctgctttggaactaatct	76	95	1
334208	3'UTR	4	2269	gaaaagtgaggcttgggttc	44	96	1
334209	3'UTR	4	2367	aaaagtctgacatggtgcaa	75	97	1

As shown in Table 1, SEQ ID NOs 20, 21, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 56, 57, 58, 60, 61, 62, 63, 64, 65, 66, 68, 69, 70, 71, 72, 73, 75, 76, 77, 78, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 94, 95, 96 and 97 demonstrated at least 40% inhibition of human diacylglycerol acyltransferase 2 expression in this assay and are therefore preferred. More preferred are SEQ ID NOs 65, 26, 29 and 35. The target regions to which these preferred sequences are complementary are herein referred to as "preferred target segments" and are therefore preferred for targeting by compounds of the present invention. These preferred target segments are shown in Table 3. These sequences are shown to contain thymine (T) but one of skill in the art will appreciate that thymine (T) is generally replaced by uracil (U) in RNA sequences. The sequences represent the reverse complement of the preferred antisense compounds shown in Table 1. "Target site" indicates the first (5'-most) nucleotide number on the particular target nucleic acid to which the oligonucleotide binds. Also shown in Table 3 is the species in which each of the preferred target segments was found.

Example 16

Antisense inhibition of mouse diacylglycerol acyltransferase 2 expression by chimeric phosphorothioate oligonucleotides having 2'-MOE wings and a deoxy gap.

In accordance with the present invention, a second series of antisense compounds was designed to target different regions of the mouse diacylglycerol acyltransferase 2 RNA, using published sequences (GenBank accession number AK002443.1, incorporated herein as SEQ ID NO: 11). The compounds are shown in Table 2. "Target site" indicates the first (5'-most) nucleotide number on the particular target nucleic acid to which the compound binds. All compounds in Table 2 are chimeric oligonucleotides ("gapmers") 20 nucleotides in length, composed of a central "gap" region consisting of ten 2'-deoxynucleotides, which is flanked on both sides (5' and 3' directions) by five-nucleotide "wings". The wings are composed of 2'-methoxyethyl (2'-MOE)nucleotides. The internucleoside (backbone) linkages are phosphorothioate (P=S) throughout the oligonucleotide. All cytidine residues are 5-methylcytidines. The compounds were analyzed for their effect on mouse diacylglycerol acyltransferase 2 mRNA levels by quantitative real-time PCR as described in other examples herein. Data are averages from three experiments in which 3T3-L1 cells were treated with the antisense oligonucleotides of the present invention. The positive control for each datapoint is identified in the table by sequence ID number. If present, "N.D." indicates "no data".

Table 2

Inhibition of mouse diacylglycerol acyltransferase 2 mRNA levels by chimeric phosphorothioate oligonucleotides having 2'-MOE wings and a deoxy gap

ISIS #	REGION	TARGET SEQ ID NO	TARGET SITE	SEQUENCE	% INHIB	SEQ ID NO	CONTROL SEQ ID NO
217299	5'UTR	11	21	ccaccctagatgagcagaaa	0	98	1
217300	5'UTR	11	36	ggtaggtagccgctgccacc	26	99	1
217301	5'UTR	11	44	agagctgaggtaggtagccg	24	100	1
217302	5'UTR	11	99	gcgctgagctccgggagctg	50	101	1
217303	5'UTR	11	183	aagccaatgcacgtcacggc	18	102	1
217304	Start Codon	11	199	gagggtcttcatgctgaagc	19	103	1
217305	Coding	11	262	gttttcgctgcgggcagctt	10	104	1
217306	Coding	11	386	gtttttccaccttagatctg	0	105	1
217307	Coding	11	403	tgagatgacctgcagctgtt	0	106	1
217308	Coding	11	447	caggccactcctagcaccag	0	107	1
217309	Coding	11	457	gatgacactgcaggccactc	29	108	1
217311	Coding	11	586	ccacacggcccagtttcgca	64	109	1
217326	Coding	11	831	gggcagatgcctccagacat	15	110	1
217327	Coding	11	841	tcggttgacagggcagatgc	31	111	1
217332	Coding	11	920	gggactcagctgcacctccc	18	112	1
217334	Coding	11	1006	cagatcagctccatggcgca	30	113	1
217335	Coding	11	1051	cacctgcttgtatacctcat	41	114	1
217340	Coding	11	1147	gaagaggcctcggccatgga	39	115	1
217344	Coding	11	1209	ggctcccccacgacggtggt	0	116	1
217345	Coding	11	1240	ggtcgggtgctccagcttgg	28	117	1
217349	Coding	11	1333	agtctctggaaggccaaatt	3	118	1
217350	Stop Codon	11	1361	ggctgggtcagttcacctcc	0	119	1
217351	3'UTR	11	1383	ctcccaggagctggcacgcg	47	120	1
217352	3'UTR	11	1424	atgcactcaagaactcggta	60	121	1
217356	3'UTR	11	1536	actgactcttcccttcttaa	39	122	1
217357	3'UTR	11	1560	acacactagaagtgagctta	57	123	1
217358	3'UTR	11	1577	cctccaccttgagcaggaca	45	124	1
217359	3'UTR	11	1599	caccaaggcccataaatatc	6	125	1
217360	3'UTR	11	1605	agaaaccaccaaggcccata	0	126	1
217361	3'UTR	11	1653	gccagggccaagtgtctgtc	46	127	1
217362	3'UTR	11	1685	tggagtcactaaggactgcc	45	128	1
217363	3'UTR	11	1715	gggacatggcctctgcctct	0	129	1
217364	3'UTR	11	1746	ggtacgaggaacccgacctg	43	130	1
217365	3'UTR	11	1772	gccagctgtgccctcagcct	0	131	1
217366	3'UTR	11	1815	ccaagccgggcagtccagat	18	132	1
217367	3'UTR	11	1861	gggtaggctcagattggaga	35	133	1
217368	3'UTR	11	1908	cggcacctgtgggacagccg	32	134	1
217369	3'UTR	11	1946	agagtgaaaccagccaacag	23	135	1
217370	3'UTR	11	2002	gctcaggaggatatgcgcca	90	136	1
217371	3'UTR	11	2033	aagcccttcctcacaccaga	9	137	1
217372	3'UTR	11	2055	ggcacctctgtgaagagaag	24	138	1
217373	3'UTR	11	2086	tcctggacccagtgtgctgc	32	139	1
217374	3'UTR	11	2124	cacacacgtgaggcttggtt	31	140	1
217375	3'UTR	11	2209	atacaaaagtgtgacatggc	30	141	1
217376	3'UTR	11	2230	tccatttattagtctaggaa	76	142	1

As shown in Table 2, SEQ ID NOs 101, 109, 114, 115, 120, 121, 122, 123, 124, 127, 128, 130, 133, 136 and 142

demonstrated at least 35% inhibition of mouse diacylglycerol acyltransferase 2 expression in this experiment and are therefore preferred. More preferred are SEQ ID NOs 142, 109 and 121. The target regions to which these preferred sequences are complementary are herein referred to as "preferred target segments" and are therefore preferred for targeting by compounds of the present invention. These preferred target segments are shown in Table 3. These sequences are shown to contain thymine (T) but one of skill in the art will appreciate that thymine (T) is generally replaced by uracil (U) in RNA sequences. The sequences represent the reverse complement of the preferred antisense compounds shown in Tables 1 and 2. "Target site" indicates the first (5'-most) nucleotide number on the particular target nucleic acid to which the oligonucleotide binds. Also shown in Table 3 is the species in which each of the preferred target segments was found.

Table 3

Sequence and position of preferred target segments identified in diacylglycerol acyltransferase 2.

SITE ID	TARGET SEQ ID NO	TARGET SITE	SEQUENCE	REV COMP OF SEQ ID	ACTIVE IN	SEQ ID NO
134026	4	579	cccaagaaaggtggcaggag	20	*H. sapiens*	143
134028	4	639	cgagactactttcccatcca	21	*H. sapiens*	144
134030	4	649	ttcccatccagctggtgaag	23	*H. sapiens*	145
134031	4	654	atccagctggtgaagacaca	24	*H. sapiens*	146
134032	4	659	gctggtgaagacacacaacc	25	*H. sapiens*	147
134033	4	664	tgaagacacacaacctgctg	26	*H. sapiens*	148
134034	4	669	acacacaacctgctgaccac	27	*H. sapiens*	149
134035	4	674	caacctgctgaccaccagga	28	*H. sapiens*	150
134036	4	679	tgctgaccaccaggaactat	29	*H. sapiens*	151
134037	4	684	accaccaggaactatatctt	30	*H. sapiens*	152
134038	4	689	caggaactatatctttggat	31	*H. sapiens*	153
134039	4	694	actatatctttggataccac	32	*H. sapiens*	154
134040	4	723	atcatgggcctgggtgcctt	33	*H. sapiens*	155
134041	4	846	gagtacctgatgtctggagg	34	*H. sapiens*	156

134044	4	909	aagaatgggagtggcaatgc	35	*H. sapiens*	157
134045	4	914	tgggagtggcaatgctatca	36	*H. sapiens*	158
134046	4	919	gtggcaatgctatcatcatc	37	*H. sapiens*	159
134047	4	924	aatgctatcatcatcgtggt	38	*H. sapiens*	160
134049	4	963	ctgagctccatgcctggcaa	39	*H. sapiens*	161
134052	4	1110	tcctggggccgatgggtcca	40	*H. sapiens*	162
134053	4	1115	gggccgatgggtccagaaga	41	*H. sapiens*	163
134054	4	1120	gatgggtccagaagaagttc	42	*H. sapiens*	164
134055	4	1125	gtccagaagaagttccagaa	43	*H. sapiens*	165
134057	4	1197	gacacctgggggctggtgcc	44	*H. sapiens*	166
134058	4	1202	ctgggggctggtgccctact	45	*H. sapiens*	167
134059	4	1207	ggctggtgccctactccaag	46	*H. sapiens*	168
134062	4	1309	ccatgtacatggaggccctg	47	*H. sapiens*	169
134063	4	1314	tacatggaggccctggtgaa	48	*H. sapiens*	170
134064	4	1319	ggaggccctggtgaagctct	49	*H. sapiens*	171
134069	4	1469	gtcatgggtgtctgtgggtt	50	*H. sapiens*	172
134070	4	1474	gggtgtctgtgggttattta	51	*H. sapiens*	173
134071	4	1479	tctgtgggttatttaaaaga	52	*H. sapiens*	174
250517	18	21985	tgaggaggatgctcttttgt	53	*H. sapiens*	175
250518	18	23110	tggactgaagcatttatagt	54	*H. sapiens*	176
250520	18	31611	tcccatccaggtaaagtgct	56	*H. sapiens*	177
250521	18	33686	catctgcctcatttcactga	57	*H. sapiens*	178
250522	18	35303	ttgatgtcacctcttttgag	58	*H. sapiens*	179
250524	18	39106	tcccttccagagctgacctg	60	*H. sapiens*	180
250525	18	37108	cccatggaggtccaggggaa	61	*H. sapiens*	181
250526	4	46	ggctgtttctctcgcgccac	62	*H. sapiens*	182
250527	4	134	gctctgcgcgaagccctggc	63	*H. sapiens*	183
250528	4	222	gcttcagccatgaagaccct	64	*H. sapiens*	184
250529	4	246	gccgcctactccggggtcct	65	*H. sapiens*	185
250530	4	441	atctcagtgctccagtgggt	66	*H. sapiens*	186
250532	4	987	gcagtcaccctgcggaaccg	68	*H. sapiens*	187
250533	4	1387	aggtgaactgagccagcctt	69	*H. sapiens*	188
250534	4	1401	agccttcggggccaactccc	70	*H. sapiens*	189
250535	4	1414	aactccctggaggaaccagc	71	*H. sapiens*	190
250536	4	1449	tgctctgtaaatttggaagt	72	*H. sapiens*	191
250537	4	1584	gcttgccctgttctaggtgg	73	*H. sapiens*	192
250539	4	1647	ttcttcccttcctgaagtga	75	*H. sapiens*	193
250540	4	1679	agtcttcttggggaagaagg	76	*H. sapiens*	194
250541	4	1707	tagtgacttggaccagttag	77	*H. sapiens*	195
250542	4	1724	tagatgattcacttttgcc	78	*H. sapiens*	196
250544	4	1763	agccacttctcatacaagcc	80	*H. sapiens*	197
250545	4	1802	acgctcgtctagtcctgaaa	81	*H. sapiens*	198
250546	4	1946	cctagtcactcatatcggag	82	*H. sapiens*	199
250547	4	1969	ggactggcctccaggatgag	83	*H. sapiens*	200
250548	4	1974	ggcctccaggatgaggatgg	84	*H. sapiens*	201
250549	4	1989	gatgggggtggcaatgacac	85	*H. sapiens*	202
250550	4	2055	gccgccaccatgagctaggt	86	*H. sapiens*	203
250551	4	2067	agctaggtggagtaactggt	87	*H. sapiens*	204
250552	4	2088	tttcttgggtggctgatgac	88	*H. sapiens*	205
250553	4	2125	tcagccttggcctggagcac	89	*H. sapiens*	206
250554	4	2137	tggagcacatgcttactggt	90	*H. sapiens*	207
250555	4	2143	acatgcttactggtggcctc	91	*H. sapiens*	208
250556	4	2150	tactggtggcctcagtttac	92	*H. sapiens*	209
250558	4	2220	ggggcctggccttctgagca	94	*H. sapiens*	210
250559	4	2242	agattagttccaaagcaggt	95	*H. sapiens*	211
250560	4	2269	gaacccaagcctcacttttc	96	*H. sapiens*	212

250561	4	2367	ttgcaccatgtcagactttt	97	*H. sapiens*	213
134018	11	99	cagctcccggagctcagcgc	101	*M. musculus*	214
134027	11	586	tgcgaaactgggccgtgtgg	109	*M. musculus*	215
134051	11	1051	atgaggtatacaagcaggtg	114	*M. musculus*	216
134056	11	1147	tccatggccgaggcctcttc	115	*M. musculus*	217
134067	11	1383	cgcgtgccagctcctgggag	120	*M. musculus*	218
134068	11	1424	taccgagttcttgagtgcat	121	*M. musculus*	219
134072	11	1536	ttaagaagggaagagtcagt	122	*M. musculus*	220
134073	11	1560	taagctcacttctagtgtgt	123	*M. musculus*	221
134074	11	1577	tgtcctgctcaaggtggagg	124	*M. musculus*	222
134077	11	1653	gacagacacttggccctggc	127	*M. musculus*	223
134078	11	1685	ggcagtccttagtgactcca	128	*M. musculus*	224
134080	11	1746	caggtcgggttcctcgtacc	130	*M. musculus*	225
134083	11	1861	tctccaatctgagcctaccc	133	*M. musculus*	226
134086	11	2002	tggcgcatatcctcctgagc	136	*M. musculus*	227
134092	11	2230	ttcctagactaataaatgga	142	*M. musculus*	228

As these "preferred target segments" have been found by experimentation to be open to, and accessible for, hybridization with the antisense compounds of the present invention, one of skill in the art will recognize or be able to ascertain, using no more than routine experimentation, further embodiments of the invention that encompass other compounds that specifically hybridize to these preferred target segments and consequently inhibit the expression of diacylglycerol acyltransferase 2.

According to the present invention, antisense compounds include antisense oligomeric compounds, antisense oligonucleotides, ribozymes, external guide sequence (EGS) oligonucleotides, alternate splicers, primers, probes, and other short oligomeric compounds which hybridize to at least a portion of the target nucleic acid.

Example 17

Western blot analysis of diacylglycerol acyltransferase 2 protein levels

Western blot analysis (immunoblot analysis) is carried out using standard methods. Cells are harvested 16-20 h

after oligonucleotide treatment, washed once with PBS, suspended in Laemmli buffer (100 ul/well), boiled for 5 minutes and loaded on a 16% SDS-PAGE gel. Gels are run for 1.5 hours at 150 V, and transferred to membrane for western blotting. Appropriate primary antibody directed to diacylglycerol acyltransferase 2 is used, with a radiolabeled or fluorescently labeled secondary antibody directed against the primary antibody species. Bands are visualized using a PHOSPHORIMAGER? (Molecular Dynamics, Sunnyvale CA).

Example 18

Effects of antisense inhibition on diacylglycerol acyltransferase 2 levels: *in vivo* studies in a diet-induced model of obesity

The C57BL/6 mouse strain is reported to be susceptible to hyperlipidemia-induced atherosclerotic plaque formation. Accordingly, these mice were fed a high-fat diet and used in the following studies to evaluate the effects of diacylglycerol acyltransferase 2 antisense oligonucleotides on mRNA expression in a model of diet-induced obesity.

Male C57BL/6 mice (7-weeks old) received a 60% fat diet for 8 weeks and subsequently received subcutaneous-injections of ISIS 217376 (SEQ ID No: 142) or a control oligonucleotide ISIS 141923 (CCTTCCCTGAAGGTTCCTCC, SEQ ID NO: 229) at a dose of 25 mg/kg twice per week for 7 weeks. ISIS 141923 is a chimeric oligonucleotide ("gapmer") 20 nucleotides in length, composed of a central "gap" region consisting of ten 2'-deoxynucleotides, which is flanked on both sides (5' and 3' directions) by five-nucleotide "wings". The wings are composed of 2'-methoxyethyl (2'-MOE)nucleotides. The internucleoside (backbone) linkages are phosphorothioate (P=S) throughout the oligonucleotide. All cytidine residues are 5-methylcytidines. A group of saline

injected mice served as untreated controls. Each treatment group contained 6-8 mice.

After the 8 week treatment period, mice were sacrificed and diacylglycerol acyltransferase 2 (DGAT2) mRNA levels were evaluated in liver, brown adipose tissue (BAT) and white adipose tissue (WAT). In addition, diacylglycerol acyltransferase 1 (DGAT1) mRNA levels were measured in these tissues. mRNA expression levels were quantitated by real-time PCR as described in other examples herein. The results are presented in Table 4 and are expressed as percent inhibition relative to saline treated mice receiving a high fat diet. A "+" preceding the number indicates that gene expression was increased, rather than inhibited.

Table 4

Antisense inhibition of diacylglycerol acyltransferase 2 expression in liver, brown adipose and white adipose tissues from diet-induced obese mice

	% Inhibition of diacylglycerol acyltransferase mRNAs					
ISIS #	Liver		WAT		BAT	
	DGAT 2	DGAT 1	DGAT 2	DGAT 1	DGAT 2	DGAT 1
141923	2	7	+26	+23	25	33
217376	80	47	87	0	78	21

The data demonstrate that diacylglycerol acyltransferase 2 antisense oligonucleotide treatment can effectively inhibit target mRNA expression in liver, brown adipose and white adipose tissue. Diacylglycerol acyltransferase 1 expression levels were slightly lowered. Although target levels were reduced, no significant changes were observed in food intake, body weight, metabolic rate or adipose tissue weight in diet-induced obese mice following treatment with diacylglycerol acyltransferase 2 antisense oligonucleotide.

Example 19

Effects of antis nse inhibition of diacylglycerol acyltransferase 2 on markers of lipid and glucose metabolism

In accordance with the present invention, ISIS 217376 (SEQ ID NO: 142) was tested for its ability to affect lipid and glucose metabolism. The diet-induced obese mice that received antisense oligonucleotide treatment, as described in Example 18, were further evaluated at the end of the 7 week treatment period for levels of serum free fatty acids, triglycerides (TRIG), cholesterol, including total cholestorol (CHOL) and high (HDL) and low (LDL) density lipoprotein cholesterol. The data, expressed as percent reduction relative to the saline control, are presented in Table 5.

Table 5

Effects of antisense inhibition of diacylglycerol acyltransferase 2 on serum cholesterol and lipids in diet-induced obese mice

	Percent Reduction in				
	Serum Lipids		Cholesterol		
ISIS #	**Free Fatty Acids**	**TRIG**	**Total CHOL**	**HDL CHOL**	**LDL CHOL**
141923	17	13	13	11	30
217376	33	41	31	28	24

The results demonstrate that antisense inhibition of diacylglycerol acyltransferase 2 expression, which was presented in Example 18, leads to significant reductions in serum free fatty acids, serum triglycerides, HDL cholesterol and total serum cholesterol. No significant change was observed in LDL cholesterol levels. With respect to glucose

metabolism, treatment with antisense oligonucleotide targeted to diacylglycerol acyltransferase 2 did reduce plasma insulin by 69% at the end of the treatment period, but did not change plasma glucose levels, glucose tolerance or insulin tolerance in diet-induced obese mice.

Example 20

Effects of antisense inhibition of diacylglycerol acyltransferase 2 on hepatic triglycerides and steatosis in diet-induced obese mice

In accordance with the present invention, ISIS 217376 (SEQ ID NO: 142) was tested for its ability to affect triglyceride and glycogen content in the livers of diet-indueced obese mice. The diet-induced obese mice that received antisense oligonucleotide treatment, as described in Example 18, were further evaluated at the end of the 7 week treatment period for hepatic triglycerides and glycogen content. Hepatic triglyceride content was used to assess hepatic steatosis, or clearing of lipids from the liver. The data are shown in Table 6 and are expressed as percent reduction relative to saline-treated, high-fat diet mice.

Table 6

Effects of antisense inhibition of diacylglycerol acyltransferase 2 on hepatic lipid and glycogen content

	Percent reduction in	
ISIS #	**Hepatic Triglycerides**	**Hepatic Glycogen**
141923	30	5
217376	56	3

The results in Table 6 demonstrate that treatment with antisense oligonucleotide targeted to diacylglycerol

acyltransferase 2 yields a marked reduction in hepatic triglyceride content compared to saline- and control oligonucleotide-treated mice, indicating an improvement in hepatic steatosis. No significant change in hepatic glycogen was observed.

Example 21

Effects of antisense inhibition of diacylglycerol acyltransferase 2 on hepatic lipogenic and gluconeogenic genes

In accordance with the present invention, ISIS 217376 (SEQ ID NO: 142) was tested for its ability to affect the expression of genes involved in fatty acid synthesis and glucose metabolism. The diet-induced obese mice that received antisense oligonucleotide treatment, as described in Example 18, were further evaluated at the end of the 7 week treatment period for expression levels of genes that participate in lipid metabolism, gluconeogenesis and glucose metabolism. mRNA levels in liver and white adipose tissue were quantitated by real-time PCR as described in other examples herein, using primer-probe sets that were generated using the GenBank accession numbers provided in Table 7. The results are presented as percent change relative to saline-treated, high fat diet control mice and are shown in Table 7.

Table 7

Lipid and glucose metabolism gene expression following antisense inhibition of diacylglycerol acyltransferase 2

		Percent Change	
Gene Nam	GenBank Accession #	ISIS 141923	ISIS 217376
Liv r tissue			

carnitine palmitoyltransferase I	NM_001876.1	-17	-49
acetyl-CoA carboxylase 1	NM_000664.1	-18	-66
acetyl-CoA carboxylase 2	NM_001093.1	-5	-90
fatty acid synthase	U29344.1	-48	-50
glucose-6-phosphatase	NM_000151.1	-27	-9
phosphoenolpyruvate carboxykinase 1	NM_011044.1	+14	+23
pyruvate kinase	NM_000298.2	-47	-73
glucose transporter type 2	NM_000340.1	-6	+8
pyruvate dehydrogenase alpha subunit	NM_000284.1	-22	-25
glycogen phosphorylase	M14636.1	-2	-19
HMGCoA reductase	NM_000859.1	-19	-45
White adipose tissue			
glucose transporter 4	M20747.1	+185	+8
glucose transporter type 2	NM_000340.1	-7	+3
hormone sensitive lipase	NM_005357.1	+75	+42
lipoprotein lipase	NM_000237.1	+113	-25

These data demonstrate that antisense inhibition of diacylglycerol acyltransferase 2, in addition to reducing the expression of target mRNA in diet-induced obese mice, is also capable of altering the expression of other genes that participate in lipid and glucose metabolism. For example, the expression levels of HMG-CoA reductase, acetyl-CoA carboxylase 1 and acetyl-CoA carboxylase 2, carnitine palmitoyltransferase I and glycogen phosphorylase, which participate in cholesterol biosynthesis, fatty acid synthesis, fatty acid oxidation and glycogen metabolism, respectively, were reduced following ISIS 217376 treatment of mice. Lipoprotein lipase, which participates in fatty acid storage in adipose tissue, exhibited reduced expression as well. Conversely, expression of enzymes that participate in gluconeogenesis, glucose-6-phosphatase and phosphoenolpyruvate carboxykinase 1, was not significantly reduced. Expression levels of hormone sensitive lipase and phosphoenolpyruvate carboxykinase 1 were significantly increased following antisense inhibition of diacylglycerol

acyltransferase.

Example 22

Effects of antisense inhibition of diacylglycerol acyltransferase 2 in the ob/ob mouse model of obesity

Leptin is a hormone produced by fat that regulates appetite. Deficiencies in this hormone in both humans and non-human animals leads to obesity. ob/ob mice have a mutation in the leptin gene which results in obesity and hyperglycemia. As such, these mice are a useful model for the investigation of obesity and treatments designed to reduce obesity.

In accordance with the present invention, the effects of antisense inhibition of diacylglycerol acyltransferase 2 were investigated in the ob/ob mouse model of obesity. Seven-week old male C57Bl/6J-Lepr ob/ob mice were fed a diet with a fat content of 10-15% and were subcutaneously injected with ISIS 217376 (SEQ ID NO: 142) or ISIS 116847 (CTGCTAGCCTCTGGATTTGA, SEQ ID NO: 230) at a dose of 25 mg/kg twice per week for 4 weeks. ISIS 116847 was used as a positive control oligonucleotide that does not target the diacylglycerol acyltransferase 2 gene. ISIS 116847 is a chimeric oligonucleotide ("gapmer") 20 nucleotides in length, composed of a central "gap" region consisting of ten 2'-deoxynucleotides, which is flanked on both sides (5' and 3' directions) by five-nucleotide "wings". The wings are composed of 2'-methoxyethyl (2'-MOE)nucleotides. The internucleoside (backbone) linkages are phosphorothioate (P=S) throughout the oligonucleotide. All cytidine residues are 5-methylcytidines. A group of saline-injected mice served as an untreated control. Each treatment group consisted of 8 mice.

At the end of the four week treatment period, the mice

were sacrificed and target expression, as well as diacylglycerol acyltransferase 1 expression, was measured in liver and fat tissue. mRNA expression was quantitated by real-time PCR as described in other examples herein. These organs were also weighed. The data are expressed as percent inhibition relative to saline control and are presented in Table 8. A "+" preceding the number indicates that gene expression was increased, rather than inhibited.

Table 8

Antisense inhibition of diacylglycerol acyltransferase 2 mRNA expression in liver and fat tissues from ob/ob mice

	% Inhibition of diacylglycerol acyltransferase mRNAs			
ISIS #	Liver		Fat tissue	
	DGAT 2	DGAT 1	DGAT 2	DGAT 1
116847	17	11	14	16
217376	83	7	90	+14

These results illustrate that treatment of ob/ob mice with an antisense oligonucleotide targeted to diacylglycerol acyltransferase 2 effectively inhibits the expression of target mRNA in both liver and fat tissues, whereas diacylglycerol acyltransferase 1 expression is not significantly changed. Liver weight was reduced by 21% in ob/ob mice treated with the antisense oligonucleotide of the present invention, but fat tissue weight was not significantly changed. No significant reduction in diacylglycerol acyltransferase 1 mRNA expression was observed.

Example 23

Effects of antisense inhibition of diacylglycerol acyltransferase 2 in ob/ob mice on serum and liver lipid

content

In accordance with the present invention, ISIS 217376 (SEQ ID NO: 142) was tested for its effect on serum lipids and free fatty acids, as well as tissue triglyceride levels in ob/ob mice.

The ob/ob mice that received antisense oligonucleotide treatment, as described in Example 22, were further evaluated at the end of the 4 week treatment period for serum lipids, serum free fatty acids, serum cholesterol (CHOL), liver triglycerides, and fat tissue triglycerides. Hepatic steatosis, or clearing of lipids from the liver, can be assessed by measuring the liver triglyceride content. The data, shown in Table 9, are expressed as percent reduction relative to saline-treated control ob/ob mice. As in Example 22, the results are the average of measurements from 8 mice.

Table 9

Serum and tissue lipid content following antisense inhibition of diacylglycerol acyltransferase 2

	% Reduction of serum and tissue lipid content				
ISIS #	Serum Lipids			Tissue Triglycerides	
	Tri-glyceride	CHOL	Free Fatty Acids	Liver	Fat
116847	22	10	8	12	14
217376	0	0	22	21	13

The data illustrate that antisense inhibition of diacylglycerol acyltransferase 2 in ob/ob mice causes a reduction in triglyceride levels in liver tissue and in serum free fatty acids. The decrease in liver tissue triglyceride content indicates an improvement in hepatic steatosis. No significant change in serum triglyceride, fat tissue

triglyceride or cholesterol was observed.

Example 24

Plasma insulin and glucose levels following antisense inhibition of diacylglycerolacyltransferase 2 in ob/ob mice

In accordance with the present invention, the ob/ob mice treated as described in Example 22 were further evaluated for insulin and glucose levels. Plasma glucose was measured at the start of the antisense oligonucleotide treatment and after 2 weeks and 4 weeks of treatment. Plasma insulin was measured following 2 weeks and 4 weeks of treatment. After 3 weeks of treatment, glucose and insulin tolerance tests were also performed in mice fasting for 16 and 4 hours, respectively. Relative to saline-treated control ob/ob mice, plasma insulin in ob/ob mice receiving ISIS 217376 was reduced by 43% at both 2 weeks and 4 weeks of antisense oligonucleotide treatment. No significant change was observed in plasma glucose levels, and glucose levels following insulin and glucose challenge were higher than in saline-treated control mice.

Example 25

Effects of antisense inhibition of diacylglycerol acyltransferase 2 in the db/db mouse model of obesity

A deficiency in the leptin hormone receptor mice also results in obesity and hyperglycemia. These mice are referred to as db/db mice and, like the ob/ob mice, are used as a mouse model of obesity.

In accordance with the present invention, antisense inhibition of diacylglycerol acyltransferase 2 with ISIS 217276 (SEQ ID NO: 142) was investigated for its ability to effect target mRNA expression, triglyceride levels and plasma glucose levels in db/db mice. Six-week old male C57Bl/6J-

Lepr db/db mice were fed a 15-20% fat diet and received subcutaneous injections of ISIS 217376 (SEQ ID NO: 142) or the control oligonucleotide ISIS 116847 (CTGCTAGCCTCTGGATTTGA, SEQ ID NO: 230) at a dose of 25 mg/kg twice per week for 4 weeks. A group of saline injected mice served as untreated controls. Each treatment group contained 4 to 8 mice.

After the 4 week treatment period, mice were sacrificed and diacylglycerol acyltransferase 2 mRNA levels (n = 4 mice) were evaluated in liver, brown adipose tissue (BAT) and white adipose tissue (WAT). Diacylglycerol acyltransferase 1 mRNA levels were also measured in these tissues. mRNA expression levels were quantitated by real-time PCR as described in other examples herein. In addition, liver triglycerides (n = 6 mice) and plasma glucose (n = 8 mice) were measured. The results are presented in Table 10 and are expressed as percent inhibition (for mRNA expression) or reduction (for glucose and triglycerides) relative to saline treated mice. An increase in gene expression or liver triglycerides is indicated by a "+" preceding the number. Hepatic steatosis, or clearing of lipids from the liver, was assessed by routine histological analysis of frozen liver tissue sections stained with oil red O stain, which is commonly used to visualize lipid deposits, and counterstained with hematoxylin and eosin, to visualize nuclei and cytoplasm, respectively.

Table 10

Effects of antisense inhibition of diacylglycerol acyltransferase 2 in db/db mice

Biological Marker Measured		Treatment	
		ISIS 116847	ISIS 217376
% R duction in plasma glucose	Week		

	0	0	0
	2	34	5
	4	55	14
% Reduction in liver triglycerides	4	+41	41
mRNA expression in tissue			
% Inhibition of diacyglycerol acyltransferase 2	Liver	+17	95
	WAT	0	80
	BAT	19	87
% Inhibition of diacyglycerol acyltransferase 1	Liver	+9	+5
	WAT	+11	5
	BAT	13	28

These data illustrate that target mRNA expression can be effectively inhibited in liver, brown adipose and white adipose tissue of db/db mice treated with the oligonucleotide of the present invention. Furthermore, inhibition of diacylglycerol acyltransferase 2 expression in db/db mice results in a reduction in hepatic triglyceride content and improved steatosis. Similar observations regarding improvement of hepatic steatosis were made in two other mouse models of obesity, the diet-induced obese mice and ob/ob mice, as described in other examples herein. No significant change in plasma glucose was observed.